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Assessment of Phytophthora sojae race population and fitness components in Iowa

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Assessment of *Phytophthora sojae* race population and fitness components in Iowa

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Signatures have been redacted for privacy

DEDICATION

To Mr. Sen Niu and Mrs Kaige Zhu who have been parents to me
For their love and caring

To my mentor Professor X. B. Yang
For his guidance, support and encouragement

TABLE OF CONTENTS

ABSTRACT

Phytophthora sojae, a homothalic Oomycete, is the causal agent of Phytophthora root rot of soybean (PRR), Oospores, the sexual spores of *P. sojae* provide the primary inoculum for the next spring. Oospores germinate when they "sense" the temperature and soil moisture level are suitable to form sporangia, which can directly germinate a growth tube to attack plants or can release zoospores, the asexual spores. Zoospore is the most important secondary inoculum.

P. sojae could attack soybean plants at any growth stage and cause severe yield loss if the infection begins early. Many surveys have been done on studying the race population distribution of *P. sojae* in different states in order to better control this disease. Change in *P. sojae* population structure over years is not uncommon. Studies were conducted in 2001 and 2002 to determine whether new races had occurred and whether the frequencies of previously existing races had changed in Iowa. In total, 19 diseased plants and 144 soil samples were obtained across Iowa. There were 19 isolates from plants and 33 from the soil that were purified, tested on eight differential lines, and classified by their virulence patterns. We found 4 new races that have not been reported previously in Iowa, races 20, 28, 35 and 41. Eight isolates were found with seven virulence formula that had not been reported. The percentage of isolates that can overcome *Rps* 1k, a widely used resistance gene in Iowa fields, has increased from 5% in 1994 to 45% in 2001 and 2002. Dominance of race 3 in *P. sojae* population on soybean plants reported in 1991 and 1994 was replaced by that of races 25 and 35. Races 1 and 3 were still the most popular races in soil in 1992 and 2001-02. The results suggested that the population composition of *P. sojae* has shifted, compared a previous survey that was completed in 1994. Race change needs to be closely monitored to provide information for effective resistance breeding to this disease.

Studies were conducted to compare fitness parameters of races of *Phytophthora sojae* isolated from Iowa soybean fields in order to explain and predict the population shift of *P. sojae.* Single-zoospore isolates of five races were cultured on V8 agar at 15°C, 20°C, 25°C, and 30°C, and colony growth rates were compared by measuring colony diameters daily for 5 days. After 6 days, eight 1-cm^2 plugs from each colony were cultured using salt washing method for zoospore production and quantification. Infection aggressiveness of *P. sojae* was determined by virulence index value (VIV), calculated by formula $VIV=1+(ab^{-1})$,

where *a* for disease incidence (%), and *b* for latent period (days/hours). Leaf disc dipping inoculation method, which involved dipping sterilized leaf discs in different zoospore suspension for 1.5 min and 15 min for the infection to occur, was adapted in the experiment. Zoospore production rates was in the order of races 35>1>3>25>28; VIV of races was in the order of races 25 > 35 > 1 > 3 > 28. Significant differences among races were observed for colony diameter, zoospore production, infection aggressiveness and one or all of these components could be used to explain the shift and might even predict the shift. Race 1 grew fastest at all 4 different temperature treatments. Together with its medium ability of spore production and infection aggressiveness, race 1 might maintain or lose its popularity in the future. Races 25 and 35 were more aggressive when infection occurred comparing to other 3 tested races and with their medium to high capacity of vegetative development and spore production, they might become the dominant races in the Iowa soybean fields. Races 3 and 28 were of the weakest fitness parameters and might further decrease their proportion in the population. Further study is needed to complete the knowledge of fitness characters of races of *P. sojae* and to predict the population shift based on the information.

GENERAL INTRODUCTION

Literature Review

Phytophthora

Oomycetes

Oomycetes is a fungal group representing an evolutionary line distinct from other fungal groups. In addition to zoosporic dispersal and oogamy, fungi in this group have cellulose containing cell walls, vegetative diploidy, heterokont flagellae (one tinsel and one whiplash), and tubular mitochondrial christae (Braisier *et al.*, 1992). They are now considered related to, and may be derived from, the heterokont algae. Supporting evidence for this relationship includes ultrastructural similarities such as flagellar rootlet structure, the mitotic apparatus, electron-dense bodies in zoospores and oogonia, and endoplasmic reticulum and cell wall microfibril structure, together with aspects of their biochemistry, such as mode of dehydrogenase regulation, cytochrome systems and oxidative metabolism, lysine sterol synthesis pathways, and mode of storage of /31-3 glucans (Braisier *et al.*, 1992; Mao *et al.*, 1996). More recent evidence involves nucleic acid sequence data. Although some researchers place the Oomycetes in the Kingdom Protoctista or Protista, this classification groups them with taxa of very different phylogeny (Hahn *et al.*, 1985). Others, such as Cavalier-Smith and Dick, place Oomycetes within a more natural group, Kingdom Chromista, with heterokont algae, diatoms, and other tinsel flagellate protists antheridia (Erwin *et al.*, 1996).

Differentiation of Phytophthora and Pythium

Almost all the various phyletic arrangements proposed for the Oomycetes distinguish a major peronosporalean line, to which *Phytophthora* belongs, from a major saprolegnialian line (Braisier *et al.*, 1992). Within the peronosporalean line, similarities between the genera *Phytophthora* and *Pythium* are widely known, and they are usually placed together in the family *Pythiaceae* on the bases of morphological similarity and details of oosporogenesis. They are distinguished primarily on the basis that *Phytophthora* differentiates zoospores in the cytoplasm before discharge from the sporangium, whereas *Pythium* first extrudes a vesicle. *Phytophthora* species also form well differentiated sporangia on distinct sporangiophores, and many species produce amphigynous rather than paragynous antheridia (Erwin *et al.*, 1996).

Until recently, only the mode of zoospore differentiation unambiguously distinguished *Phytophthora* from *Pythium*, and even this distinction is sometimes vague. However, growing molecular evidence now supports the separate status of the two genera. Some monoclonal antibodies distinguish *Phytophthora* from *Pythium. Phytophthora* species examined lack an inverted repeat sequence of mitochondrial DNA found in *Pythium* and other Oomycetes (Braisier *et al.*, 1992). DNA sedimentation rates also differ in the two genera, and *Pythium undulata* has recently been transferred to *Phytophthora* on this basis and that of zoospore differentiation. A preliminary comparison of 28S ribosomal RNA sequences in 23 species of *Phytophthora* and *Pythium* indicates that the two genera share a common root, and much greater genetic differences are found between them than within them (Braiser *et al.*, 1992; Faris *et al.*, 1992; Förster *et al.*, 1989). The *Pythium* species also fall into three clusters and exhibit much more diversity than the rather closely grouped *Phytophthora* species, suggesting that *Phytophthora* is of more recent origin than *Pythium.*

Phytophthora sojae

Classification of P. sojae

P. sojae, a homothalic Oomycete, is the causal agent of Phytophthora root rot (PRR) of soybean, *Glycine max*. The pathogen responsible for the disease was first attributed to *Fusarium* or *Diaporthe*. Suhovecky and Schmitthenner (1955) isolated *Phytophthora* from diseased plants. In 1957, Herr reported that *Phytophthora cactorum* was the pathogen of PRR. Kaufman and Gerdemann (1958) carried out the first comprehensive study of PRR and found the pathogen was different from two closely related *Phytophthora* species, *P. cactorum* and *P. megasperma*, in morphology, pathogenicity, and vegetative growth rate. They named the pathogen *P. sojae*. One year later, Hildebrand (1959) addressed that the pathogen should be in the category of *P. megasperma*. Because of the high host specificity of the pathogen, he suggested to rename it *P. megasperma* var. *sojae*. In 1980, Kuan and Erwin compared *P. megasperma* isolates from a variety of hosts and found there was no significant difference in the sizes of oospores of different isolates. They thought it was inappropriate to classify variety of *P. megasperma* based on oogonia size. They also found that the isolates from soybean and alfalfa, *Medicago savita*, could only cause diseases on their own hosts, so they suggested naming these two pathogens *P. megasperma* f. sp. *glycinea*, and *P. megasperma* f. sp. *medicaginis*. Hansen and Maxwell (1991) compared *P. megasperma* isolates in morphology, protein pattern with polyacrylamide gel

electrophoresis, isoenzyme patterns (Nygaard *et al.*, 1989), nuclear DNA amount, chromosome number, restriction DNA on mitochondria (restriction fragment length polymorphism, RFLP), pathogenicity, and growth rate to separate the *P. megasperma* population into four species: *P. sojae*, *P. medicaginis*, *P. trifolii*, and *P. megasperma.* After 30 yr of research, classification of the causal agent of PRR was finally given the name of *P. sojae* Kuaf. et Gerd, which was initially suggested by Kaufmann and Gerdemann in 1958.

Morphology and biology of P. sojae

P. sojae grows slowly on PDA. The colony is white, with dense cottony, patternless growth to the colony margin. Colony on cornmeal agar, lima bean agar, or V-8 juice agar grows faster than on PDA and has an even margin. The mycelium is 3–9 µm in width and coenocytic when young, becoming septate with age (Suhovecky *et al.*, 1955). Sporangiophores are simple and determinate. Sporangia are terminal, obpyriform, and nonpapillate, with a size of 42–65 x 32–53 µm (Braisier *et al.*, 1992). Zoospores are extruded from sporangia; they are ovoid, bluntly pointed at both ends, and have two flagellae, one at either end. Cysts either form zoospores or germinate directly by producing appressoria. Oogonia are thin-walled and spherical with a 29–58-µm diameter. Oospores have thick, smooth inner and outer walls.

P. sojae is a soilborne pathogen. Oospores are the primary inoculum source for *P. sojae* (Athow, 1985). Massive numbers are produced in roots during epidemics. They can survive in crop residues and soil for many years, but they do not grow competitively or colonize soil debris. *P. sojae* also can survive as mycelium or oospores in the seed coat.

Different *P. sojae* pathotypes (races) probably evolved independently from the same clonal lineage, because it is homothallic and a poor correlation was found between RFLPs (Whisson *et al.*, 1994), mitochondrial DNA polymorphisms (Förster *et al.*, 1989), and pathotypes. However, *P. sojae* does outcross at low frequencies (Förster *et al.*, 1994), and some of the pathotypes may have been created as a result of outcrossing, although mutation is thought to be more important for creating diversity (Bhat *et al.*, 1993; Förster *et al.*, 1994).

Phytophthora root rot symptoms

P. sojae can attack any part of soybean plants at any growth stage (Suhovecky *et al.*, 1955). Disease development is most rapid at soil temperatures above 15.56°C with high soil moisture. This disease is most common in low-lying areas of a field, in poorly drained or compacted soils, and in soils with high clay content. *P. sojae* can cause seed rot, and a

laboratory test is needed to distinguish it from other seedling pathogens such as *Pythium* species. For infection in the late growth stages, symptoms could include yellowing; wilting; and death of plants; or stunting with no death of plants, which could be confused with flood damage. However, examining the root tissue of soybean plants can differentiate the two causes of death because flood damage only destroys the cortical cells of the roots and leaves the root center intact, whereas *P. sojae* grows in all parts of the soybean roots and turns the root tissues tan to dark brown. The symptoms for diagnosing PPR on soybean plants are described below.

Seeds and seedlings. Infected seeds become rotten, decayed, and die. Seedlings from seeds infected by *P. sojae* can turn dark brown or black, and eventually die (Yang *et al.*, 1996). Diseased young seedling stems close to the ground can have water-soaked lesions, yellowing, and wilted leaves, and the whole seedling can suffer from soft rot and collapse of the root system.

Roots. One of the key differences between PRR and other stem rot diseases is little or no roots are left on plants attacked by *P. sojae* (Draper *et al.*, 2001). Roots show a brown discoloration on the surface and, if split, the inner tissues show a tan to brown discoloration.

Stems. A key diagnostic symptom of Phytophthora stem rot is the brown lesion that develops from the roots and progresses several nodes up the stem from the soil line (Yang *et al.*, 1996). Sometimes, the *Phytophthora* lesion may develop on only one side of the soybean stem. Stems can be cut open to determine whether the internal tissues are colonized from the soil line to the top of the lesion to diagnose the disease.

Leaves. Foliar symptoms on older plants occur as general yellowing of the lower leaves that progresses upward on the plant, followed by wilting and death (Draper *et al.*, 2001).

Pods. Diseased plants can eventually be killed during a growing season before forming pods. Soybean yield can be significantly reduced if severe disease occurs (Schimitthenner *et al.*, 1985). Even if the plants survive, they produce fewer and smaller pods. When the plants are infected in late growth stages, water-soaked lesions form on the bottom of young pods. The lesion elongates to the top of the pod and the color turns darker until the entire pod becomes yellowish brown with dried, diseased seeds. The pathogen can survive in the seed coat and be long-distance transported.

Disease cycle of soybean root rot caused by P. sojae

The life cycle of *P. sojae* is shown in Fig. 1.1. Oospores, the sexual spores of *P. sojae*, overwinter in the soil, on plant debris, or even on the seed coat of soybean seeds, and they provide the primary inoculum for the next spring (Faris *et al.*, 1992). Oospores can survive in the soil for many years even if the plant residues have decomposed (Xu *et al.*, 1998). They germinate when they "sense" the temperature and soil moisture level are suitable to form sporangia. Sporangia can either indirectly form zoospores and release them when the soil is flooded or saturated, or they can directly attack plants by germinating a growth tube and mycelia. Chemicals such as genistein and other isoflavanoid exudates produced and released by hosts can attract biflagellate zoospores, swimming toward the hosts. After reaching the root or seed surface, zoospores lose motility and form germ tubes, develop mycelia, and produce more sporangia, and more zoospores (Hickman *et al.*, 1966). Zoospores can be produced in abundance on host tissue when environmental conditions are favorable and play the important role of secondary infection inoculum. *P. sojae* is homothetic and needs only one mating type to produce sexual spores, or oospores (Schmitthenner 1988, 1989).

Phytophthora root rot

Mechanisms of dispersal

Dispersal can be defined as movement of individuals or their propagules into or out of the population or population area. *P. sojae* can spread as oospores, mycelia, and zoospores to adjacent fields by farm management practices such as tillage and irrigation (Schmitthenner 1988, 1989; Thompson *et al.*, 1994; Workneh *et al.*, 1998). Long-distance dispersal of *P. sojae* is mainly attributed to human activities, including transporting contaminated seeds, plant propagules, or soil (Ristaino *et al.*, 2000).

Distribution and yield loss of P. sojae worldwide

P. sojae, the casual agent of Phytophthora root rot of soybean, is widely distributed (Fig. 1.2). It presents in countries in Asia, Australia, Europe, North America, and South America. There are no reports from Africa on *Phytophthora* presence. This disease has drawn the attention of American researchers since it was first discovered. In 1998, the total yield loss of the top 10 soybean-producing countries caused by PRR was 1,266 thousand metric tons, and the loss in the United States was 1,149 thousand metric tons, more than 10 times that of the second most severely infected country, Argentina, which lost 92 thousand

metric tons (Wrather *et al.*, 2001a) to this disease. It is widespread in the United States, where state records document its development historically.

Distribution and yield loss in the United States

Until 1996, soybean was widely grown from southern Texas to northern North Dakota and from the east coast to western Texas (Yang and Feng, 2001). In total, 33 soybean diseases have been documented since 1996. Phytophthora root and stem was the most damaging disease, second only to soybean cyst nematode. This disease distributes across 2,239.41 km from west to east, and 1,623.93 km from north to south in North America (Yang and Feng, 2001), and more than 23 states have reported the presence of this disease (Fig. 1.3). Estimated yield loss in southern United States due to PRR was 1.04 to 2.83% of the total yield loss annually from 1985 to 1993 (Mulrooney, 1988a,b; Sciumbato, 1993; Wrather *et al.*, 1995a), approximately $6 million loss every year, and averaged 1.08% of the total yield loss from 1974 to 1994 (Wrather *et al.*, 1995b, 2001b).

The North Central Region is called the "Soybean Belt" in the United States. Ten states in this region are in the top 12 production states and six are the most productive states. According to Doupnik (1993), the North Central Region produced approximately 80% of the total soybean production of United States. From 1989 to 1991, the average disease loss on soybean in the North Central Region was 13.17% of the total yield, which was an approximate $1,302 million (calculated based on $5.5/bu) loss. Among the estimated disease loss, PRR caused $187.952 million yield loss every year, followed by soybean cyst nematode. This disease plays a major role in limiting soybean production in the north central United States. Effective monitoring and management systems need to be developed and implemented.

Phytophthora race distribution history in the North Central Region

P. sojae was detected in 67% of the soybean fields in Ohio and Minnesota, 63% of Iowa fields, 55% of Missouri fields, and 41% of Illinois fields by 2002 (Ohio was the second state from which PRR was identified; Schmitthenner, 1989). It has been the most destructive disease of soybean in the state. More than 70 races of *P. sojae* have been detected in Ohio soils. *P. sojae* has been recovered from 82 of 86 fields taken from 20 Ohio counties (Dorrance *et al.*, 2003). Leitz *et al.* (2000) reported that most of the recovered isolates were classified to races 1, 3, and 4, or variants with the addition of *Rps1d* virulence in Illinois. Races 41, 43, 54, and 55 also were recovered from their survey. *P. sojae* race surveys also were identified in Nebraska, and 55 races were identified by 2000. As a result of ongoing

survey efforts, races 1, 3, 4, 25, and 28 have been recovered from Nebraska soybean fields. Previous surveys were conducted in eastern Nebraska in 1980 and 1981 and resulted in recovery of races 1, 3, 9, 14, 18, and 23 (Giesler *et al.*, 2002). During an assessment (Safir *et al.*, 1997) from 1993 to 1996, races 3, 4, 11, and 15 and 10 new races were found in Michigan, whereas races 1, 3, 4, 7, 11, and 25 were the races identified from a previous study. Kaitany *et al.* (2001) recovered races 2, 41, and 44 for the first time in Michigan fields. Population shift has not just occurred in Michigan; shifts have been found in many states.

In Iowa, race 1 was the only race found before 1966 (Tachibana *et al.*, 1975). Yang *et al.* (1996) conducted a race assessment of *P. sojae* and identified races 1, 2, 3, 4, 8, 13, 15, and 25. Race 3 was the dominant race from diseased plant samples in their study, and race 1 was the dominant race recovered from soil samples. Since then, no further survey was conducted to monitor the population shift.

PRR management strategies

There are six methods to control PRR. None of them works very effectively when used individually. Integrated management is required in this disease system.

Fungicide seed treatment

In areas where Phytophthora root rot is a consistent problem, fungicide seed treatments can be used to reduce the early season damping-off. Metalaxyl or mefenoxam (Metalaxyl-M) is the active ingredient of most of the seed treatment fungicides (Dorrance *et al.*, 2001). A list of products containing these compounds and their recommended rate of application is provided in Table 1.1 (Draper *et al.*, 2001).

Improve soil drainage, aeration, and structure

Wet and waterlogged soils provide a favorable environment for many soilborne pathogens, including *P. sojae* (MacDonald *et al.*, 1994). Compacted soils also promote wet soils for longer periods and promote disease development (Duniway *et al.*, 1983; Workneh *et al.*, 1998, 1999). An essential step to control them is to improve soil drainage so that flooding is eliminated or minimized. However, excess tillage should be avoided if this activity compacts the soil. Improving drainage is particularly important in no-tillage soils that retain moisture and require less precipitation to saturate the soil. *Phytophthora* zoospores are produced only in saturated soil (Schmitthenner, 1988); if soils are not saturated early in the season, varieties with partial resistance escape disease and remain disease-free throughout the season (Schmitthenner *et al.*, 1985). To control *Phytophthora* damping-off, especially in

poorly drained fields to be planted no-tillage, higher rates of these fungicides should be applied.

Crop rotation

Because oospores can survive in the soil for long periods, overuse of soybean can increase inoculum levels and promote the development of new races (Schmitthenner *et al.*, 1985, 1994). Crop rotation prevents the rapid buildup of inoculum but does not eliminate the disease or eradicate *Phytophthora* (Duniway *et al.*, 1983; Workneh *et al.*, 1998, 1999b). Under high levels of inoculum due to the repeating use of soybean, the effectiveness of partial resistance declines.

Resistant cultivars

Choosing the right cultivar is extremely important when attempting to manage Phytophthora root rot (St. Martin *et al.*, 1994). The susceptible variety achieved 65–69% of the yield of the resistant cultivar, and blends of susceptible and resistance cultivars could have 83–89% of the yield of the resistance cultivars (Wilcox *et al.*, 1998). There are two different types of genetic resistance in soybean varieties. Race-specific resistance is effective against certain races of the pathogen (Slusher *et al.*, 1973; Jee *et al.*, 1998). The resistance genes are designated as *Rps* (resistant to *P. sojae*) genes (Schmitthenner, 1988). The second type is partial resistance, also called field resistance or tolerance. Partial resistance is effective against all races of *P. sojae*, but the level of resistance is not complete, so some level of disease does occur with moderate influence on yield (Slusher *et al.*, 1973; Schmitthenner *et al.*, 1985; Schmitthenner 1988, 1989). In problem fields, it is best to choose varieties with race-specific resistance combined with partial resistance to achieve better levels of control (St. Martin *et al.*, 1998). Race-specific resistance varieties are available with resistance genes *Rps* 1, *Rps* 1b, *Rps* 1c, *Rps* 1d, *Rps* 1k, *Rps* 3a, *Rps* 6, and *Rps* 7, and gene combination varieties such as *Rps* 1k+3a or *Rps* 1c+3a, and *Rps* 1b+3a (Yang *et al.*, 1996). Ohio State University Extension reported an increasing number of fields in Ohio in which single *Rps* genes of *Rps 1k* or *Rps 1c* will not be effective due to the adaptation of the pathogen to these *Rps* genes (Dorrance *et al.*, 2001). Disease management in fields with severe *Phytophthora* problems needs combination resistance gene varieties or a higher level of partial resistance.

Monitor *Rps* gene performance

Performance of the resistance package in soybean varieties needs to be closely monitored. When optimum disease conditions develop later in the growing season, scout

those areas of the fields to look for stem rot development. If a large number of plants with Phytophthora stem rot are found, varieties with different resistance genes and higher partial resistance should be adopted after determining the prevalent race(s) in the fields.

Monitor population shifts of P. sojae

Fifty-five races of *P. sojae* have been identified (Giesler *et al.*, 2002). Race population shifts have been observed in many states within the United States, as well as in other countries (Anderson *et al.*, 1992; Ryley *et al.*, 1998; Leitz *et al.*, 2000; Kaitany *et al.*, 2001; Dorrance *et al.*, 2003). Occasional crosses and mutations of the pathogen cause new races to occur in the field (Bhat *et al.*, 1993; Förster *et al.*, 1994; Drenth *et al.*, 1996). Under selective pressure, races that can overcome more resistance genes will become more prevalent. It is unclear how long a single *Rps* gene or a few stacked *Rps* gene will remain viable; the race population shift needs to be carefully monitored to ensure the effective use of resistant cultivars.

Thesis organization

This thesis consists of a general introduction with literature citations as the first chapter, two separate chapters as journal manuscripts, and a summary. Tables and graphs are attached with each chapter.

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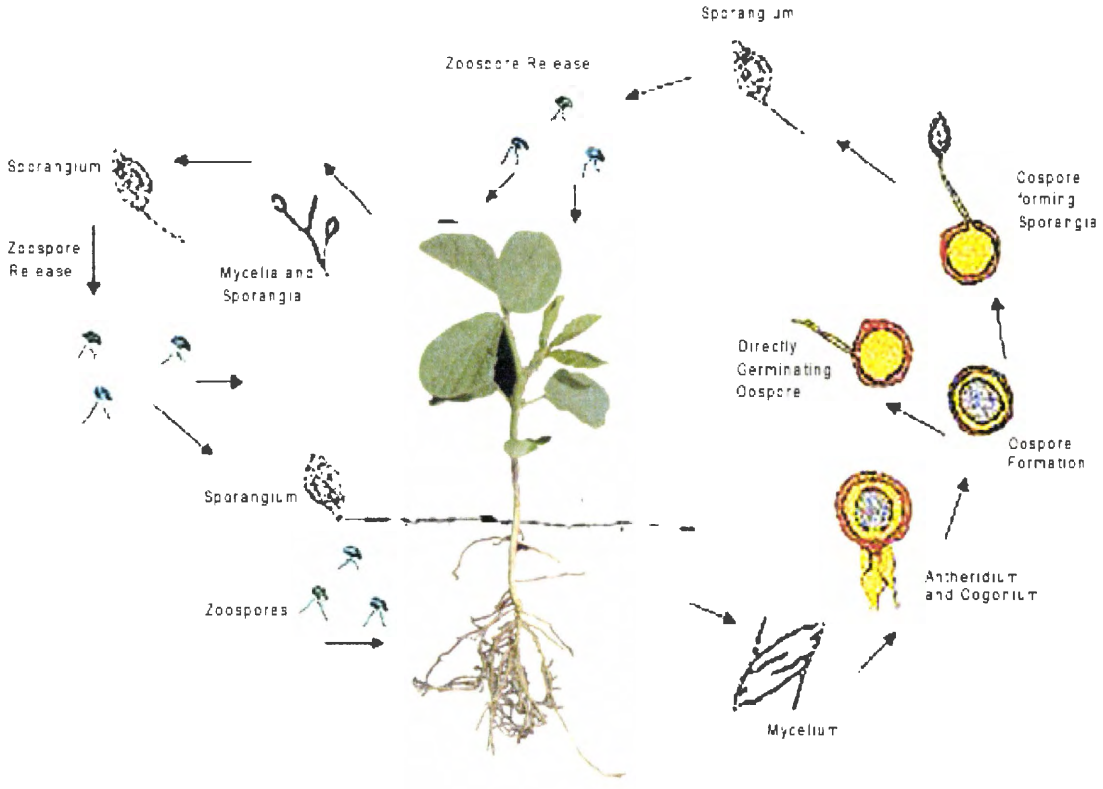

Fig. 1.1. Disease cycle of soybean root and stem rot caused by *P. sojae.*

Fig. 1.2. Distribution map of Phytophthora root and stem rot of soybean (casual agent *P. sojae*) (resource: CABI compendium).

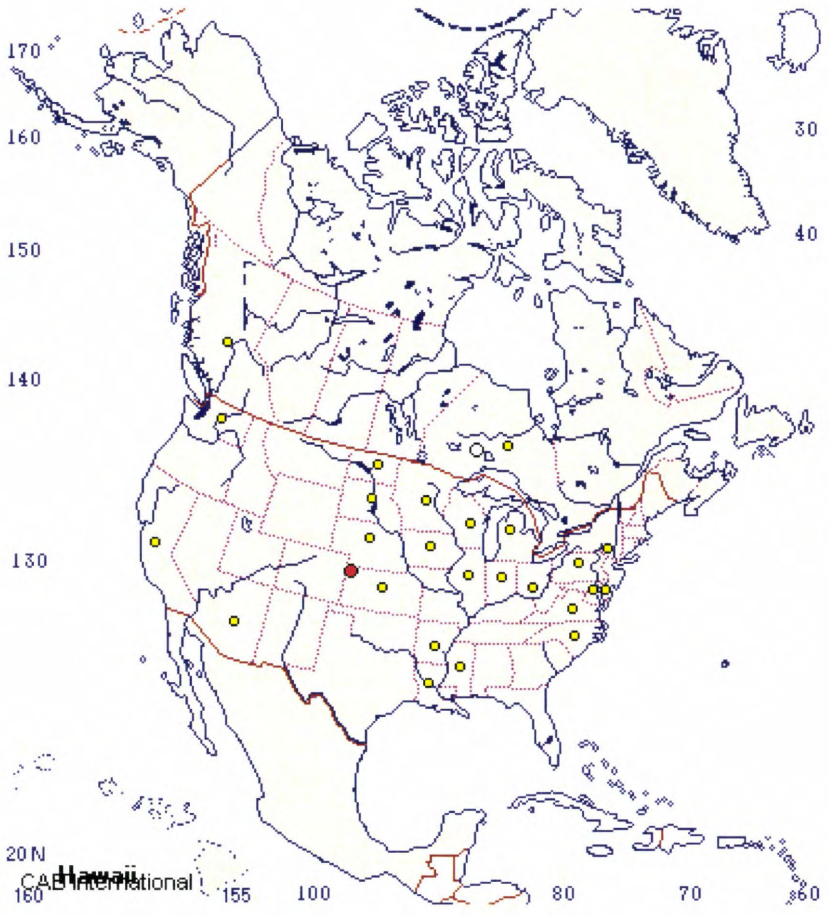

Fig. 1.3. Distribution map of North American Phytophthora root and stem rot (casual agent *P. sojae*) (resource: CABI compendium).

Table 1.1. Seed treatment fungicides labeled for *P. sojae* control on soybean

Product name	Common name	Manufacturer
Allegiance LS	Metalaxyl	Gustafson
Allegiance FL	Metalaxyl	Gustafson
Allegiance Dry	Metalaxyl	Trace Chemicals LLC
Apron FL	Metalaxyl	Gustafson
Apron Flowable	Mefenoxam	Wilber-Ellis
Apron Maxx RTA	Mefenoxam and Fludioxonil	Syngenta
Apron Maxx RTA + Moly	Mefenoxam and Fludioxonil	Syngenta
Apron TL	Metalaxyl	Wilber-Ellis
Apron XL LS	Metalaxyl	Syngenta
System 3	Pentachloronitrobenzene (PCNB), Metalaxyl, and *Bacillus subtilis* GBO	Helena
Warden RTA	Mefenoxam and Fludioxonil	Agriliance LLC

(Draper *et al.*, 2001).

CHANGES IN *PHYTOPHTHORA SOJAE* RACES IN IOWA SOYBEAN FIELDS

Abstract

Phytophthora causes soybean Phytophthora root rot and is an important disease in Iowa soybean, *Glycine max*. Before 1994, race 25 of *Phytophthora sojae* was not considered a key threat because of its low frequency among the races found in Iowa, although it can defeat the widely used resistance gene *Rps* 1k. Studies were conducted in 2001 and 2002 to determine whether new races had occurred and whether the frequencies of previously existing races had changed in Iowa. In total, 19 diseased plants and 144 soil samples were obtained across Iowa. Isolations were made using a leaf disc baiting techniques. Nineteen isolates from plants and 33 from soil were purified, tested on eight differential lines, and classified by their virulence patterns. We found three new races new to Iowa. The new races were 20, 28, and 35. Eight isolates were found with seven virulence formula that had not been published previously (*Rps* 1c; *Rps* 1b, 1d, 3a, 6; *Rps* 1a, 1b, 1c, 1d, 1k, 3a, 7; *Rps* 1a, 1c, 1d; *Rps* 1a, 1b, 1c, 1d, 1k, 6, 7; *Rps* 1a, 1b, 1c, 1k; and *Rps* 1a, 1b, 1d, 1k, 6, 7). These isolates could be new races that have not been reported in the United States. The percentage of isolates that overcame *Rps* 1k has increased significantly ($P < 0.05$) from 5% in 1994 to 45% in 2001 and 2002. Dominance of race 3 in *P. sojae* population on soybean plants reported in 1991 and 1994 was replaced by that of races 25 and 35. Races 1 and 3 were still the most popular races in soil in 1992 and 2001–2002. The results suggested that the population composition of *P. sojae* has shifted, compared with a previous survey that was completed in 1992–1994. Race change needs to be closely monitored to provide information for effective resistance breeding to this disease.

Introduction

The soilborne pathogen *Phytophthora sojae* M.J. Kaufmann & J.W. Gerdemann (Syn. *P. megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin) is the causal agent of Phytophthora preemergence damping-off, and root and stem rot of soybean (PRR). Poor soil drainage, conservation tillage, and lack of crop rotation can influence the soil environment in ways that favor PRR disease development (Workneh *et al.*, 1998, 1999). *P. sojae* has reduced the yield of soybean in the United States, 1,149 thousand metric tons,

more than 10 times that of the second most severely infected country, Argentina. According to the report of Doupnik (1993), the North Central Region produced approximately 80% of the total soybean production of the United States. From 1989 to 1991, PRR caused approximately $188 million in yield loss in the North Central Region annually. Estimated yield loss in the southern United States from PRR was from 1.04 to 2.83% of the total yield loss annually from 1985 to 1993 (Mulrooney, 1988a,b; Sciumbato, 1993; Wrather *et al.*, 1995a), approximately $6 million loss every year, and averaged 1.08% of the total yield loss from 1974 to 1994 (Wrather *et al.*, 1995b, 2001b).

In the United States, soybean PRR was first found in Indiana in 1948 and subsequently it was found in Ohio in 1951 (Schmitthenner, 1989). Since then, more states have reported the presence of PRR. This disease is distributed across 2,239.41 km from west to east, and across 1,623.93 km from north to south in North America (Yang and Feng, 2001). More than 23 states have reported the presence of this disease.

Recently, a series of studies have been conducted in the North Central Region to monitor the race population of *P. sojae*. More than 70 races of *P. sojae* have been detected in Ohio soils. *P. sojae* was recovered from 82 of 86 fields in 20 Ohio counties. Isolates that could defeat the *Rps* 1k gene, which confers PRR resistance, increased from 20% during 1990 and 1991 to more than 50% during 1997 to 1999 (Schmitthenner *et al.*, 1994b; Dorrance *et al.*, 2003). Leitz *et al.* (2000) reported that most of the recovered isolates were classified to races 1, 3, and 4, or variants with the addition of *Rps* 1d virulence in Illinois. Races 2, 3, 4, 25, 41, and 44 were recovered from plant samples during 1993 to 1997 (Kaitany *et al.*, 2001) in Michigan, and where only races 3 and 4 had been reported before this study (Lockwood *et al.*, 1978). Fourteen races, and eight new races, 46 to 53, were identified in Australia during 1979 to 1996 (Ryley *et al.*, 1998), whereas only race 1 was found in Argentina from 1989 to 1992 (Barreto *et al.*, 1995). These studies have shown that the occurrence and prevalence of *P. sojae* races vary geographically. States with longer presence of *P. sojae* have more diversity than those states where *P. sojae* occurrence is more recent.

Tachibana *et al.* (1975) found only *P. sojae* race 1 in Iowa in 1966, and no other races were found in their survey, which was conducted from 1966 to 1973. In a 1991–1994 survey (Yang *et al.*, 1996), the situation had changed dramatically. *P. sojae* had become the major causal fungus associated with the seedling disease complex of soybean in Iowa. In

1994, 24.3% of the diseased seedling samples were infected by *P. sojae* (Rizvi *et al.*, 1996). Races 2, 3, 4, 8, 13, 15, and 25 were found by Yang *et al.* (1996) in addition to race 1.

Race 25 is of great concern because it can overcome the resistant gene *Rps* 1k gene, which is widely used in soybean cultivars and is resistant to 21 races (1-11, 13-15, 17, 18, 22, and 24). The gene has a broad resistance spectrum and was effective in 99% of fields in Wisconsin (Grau, 2003). In Ohio soybean fields, the percentage of isolates that could overcome *Rps* 1k increased from 20% to more than 50% in 6 to 7 yr (Dorrance et al., 2003). Cultivars with *Rps* 1k have been planted widely since 1981 in Indiana. Races 1 and 3 once were the prevalent in Indiana fields; however, more races were recovered and virulent to *Rps* 1k. During 1990 to 1993, four of the seven new races found in Indiana were virulent on soybean with the *Rps* 1k gene (Abney *et al.*, 1997). Michigan researchers found that varieties with *Rps* 1k were only 69% resistant to all the isolates recovered during 1993 to 1997 (Kaitany *et al.*, 2001). In Yang *et al.* (1996), 4.6% of the plant samples were affected by race 25 in Iowa in 1991.

In 1994, Schmitthenner *et al.* (1994b) proposed that severity of PRR on soybean cultivars with the *Rps* 1k gene would increase within a few years. This disease provided a challenge to plant pathologists to detect the loss of resistance of specific resistance genes to the pathogen population and to determine the economic threshold in which single gene deployment is no longer effective because of the defeat of the resistance of cultivars to *P. sojae*. An effective monitoring and management system is needed. No research has been conducted since the Yang *et al.* (1996) survey during 1991 to 1994. The present survey was designed to detect the presence and distribution of races of *P. sojae* isolated from soil or diseased soybean plants in Iowa

Material and methods

Plant samples

In summer 2001, soybean plants with PRR symptoms were arbitrarily collected from soybean fields in 10 Iowa counties. Each sample contained two to four plants. All counties were located in central Iowa, except for Lee County (southeastern Iowa). Another two plant samples, three plants for each sample, were sent by farmers to Iowa State University Plant Pathology Clinic in spring 2002. The number of fields sampled per county ranged from one to five. Three to five plants were collected at each field and constituted a

sample. Plant samples were placed in plastic bags and stored for a maximum of 2 weeks at 4°C to maintain viability of the pathogen before processing.

Plants were washed with tap water for 1 h to remove soil, leaves, and any loose plant debris. After removing the loosened surface tissue with a razor blade, the plants were surface sterilized by rinsing in 70% ethanol for 5 s, and then sterilized in distilled water. After drying on sterilized paper towels in a fume hood, roots were segmented into small pieces approximately 0.8–1.2 cm in length. After the root pieces were placed on Masago agar (Schmitthenner *et al.*, 1994a), the media were inverted and covered the pieces. The plates were labeled and incubated in the dark at room temperature. Small white colonies, which tended to grow on the surface of the medium and into the air because of the antibiotics in the Masago medium could be observed 4 d after transferring. Colonies were white and had a cotton-like mycelium as *P. sojae* were transferred to Masago agar followed by dilute V-8 juice agar (Schmitthenner *et al.*, 1994a) to be purified by transferring a small piece of mycelia from the edge of the colony. The last transfer step was repeated until the colony become pure. Enzyme-linked immunosorbent assay (ELISA) kits (Neogen Co. & Agdia Co.) were used to confirm that the isolates were *P. sojae*.

Soil samples

In summer 2001, 90 soil samples were collected from 77 Iowa counties. Locations of the sample fields are shown in Fig. 2.1. Sample fields were determined arbitrarily before the collection. They were selected to represent soybean production area in Iowa. Fields with low spots were intentionally selected because *P. sojae* tends to accumulate around low spots that usually have free water or poorly drained soil.

A zigzag sampling route was taken while sampling, and samples were collected from 10 points on the pattern in each field by using a small shovel. Soil from each spot weighed approximately 0.075 kg. Soil taken from the 10 points was mixed thoroughly after removing the large plant debris, sealed in labeled paper bags, and treated as one sample. Each sample weighed approximately 0.75 kg.

Soil samples were blown dry in a transfer hood and stored in cool room until needed. The whole sample preparation procedure was performed in a greenhouse at 29–30°C. *P. sojae* was isolated from the soil by using a leaf disc baiting technique. The bottom of each of 90 7.6-cm-diameter plastic cups was punctured with three 1.3-cm-diameter holes. Each plastic cup was filled with one soil sample to 80% full. All the cups were watered until

saturation daily for 4 d. Water was generously poured into the cup from the edge, without disturbing the soil surface. After 4 d in a shaded area, the cups were set into cups with no holes, and watered until saturation, and kept for another 24 h. During the 24 h, a 1-cm layer of water was kept above the soil. Twelve 6-mm-diameter leaf disks ('Sloan') were floated on the water. After incubating for 24 h, the leaf disks were taken back to the laboratory, surface sterilized with 5% Clorox for 30 s, dried on sterilized paper towels, and plated onto Masago media. Paper towels were wrapped in aluminum foil and autoclaved for 30 min. Plates were incubated in darkness at room temperature (23-25°C). After 4 d, small white colonies became visible on the edge of the leaf discs. Colonies were pure white, with a cotton-like mycelium that grew mostly on the surface of the medium into the air. Colonies that had these characteristics described above were transferred to Masago agar followed by dilute V-8 juice agar to be purified by transferring a small piece of mycelium from the edge of the colony. The transfer and purification step were repeated until pure cultures were obtained. Neogen and Agdia enzyme-linked immunosorbent assay (ELISA) kits were used to confirm whether the isolates were *Phytophthora*.

ELISA test

Two types of kits were used, a small-quantity kit that contained eight microwells and a large-quantity kit that contained 96 microwells. ELISA kits of both large and small quality contained positive control solution, negative control solution, extraction solution, wash solution, anti-*Phytophthora* peroxidase conjugate, substrate concentrate, substrate diluent, and stop solution. No preparation was necessary for the eight-microwell kits except sample preparation. For the 96-microwell kits, both sample preparation and working solution preparation were needed. All the solutions and samples were prepared freshly on the day of use. The complete test procedure was carried in a transfer hood followed the instruction provided by company where ELISA kits were obtained. Solution in negative control wells produced no color, whereas the solution in positive control wells was green or blue, depending on the way the kit was designed. For the sample wells where the solution turned color, corresponding isolates were considered as *Phytophthora*. Purified and ELISA-tested isolates were transferred to water agar and incubated in the dark at room temperature for 7 d. Agar plugs approximately 1 x 2 cm large were taken from the edge of the colony, and two water agar plugs with mycelium were transferred into each tube and stored in the water for long-term storage.

Race classification

Differential cultivars

Nine varieties were used in the race test. They were the same varieties that Yang *et al.* (1996) used in their 1994 assessment, except for two. Compared with 1991–1993 study, they used four varieties (Table 2.1). Twelve to 14 seeds of each cultivar were planted in each 10.2-cm-diameter clay pot. Each isolate had three replication tests on each cultivar; therefore, 27 pots of seedlings were used for testing each isolate. Pots were filled with sterilized mixture of peat, perlite, and soil (1:2:1) until 75% full. Seeds were placed above the soil, and at a 2-cm depth of soil was used to cover the seeds. The pots were watered until saturation. Pots were then incubated on a greenhouse bench in random order at 29–30°C with direct lights provided. Plants were irrigated daily by the greenhouse manager by using tap water.

Inoculation

P. sojae isolates were transferred to oatmeal agar in laboratory 2 d after the seeds were planted, sealed, and incubated in the dark at room temperature until needed. The inoculation procedure was taken place approximately 8–10 d after the planting. A piece of paper was wetted with tap water and placed on the cover of a crisper. The plates were unsealed and the oatmeal agar with the mycelium was inverted on the wet paper towel. A razor blade was used to carefully remove the medium as much as possible and leave a thin layer of mycelium for inoculation. Five healthy seedlings of similar size were inoculated per pot. The sharp point of a straight blade was used to create a 3-mm-long wound on the hypocotyls of the seedlings and a small piece of mycelium was inserted, which was approximately 3 mm in length, into the wound. The same technique was used to inoculate all the plants.

Inoculated seedlings were incubated in a dew chamber for 24 h to provide moisture and time for *P. sojae* to infect the wounded plants. The chamber was preset with 30°C water temperature, 20°C wall temperature, and 28 to 30°C of air temperature and allowed to sit for 6 h before using. No light was provided during the dew chamber incubation.

After incubation, the plants were taken out of the chamber, moved back to the greenhouse bench, randomly arranged, and provided with direct light. Plants were placed in the light for 72 h to recover from the darkness before the evaluation.

Data collection

Each isolate had three replications for one cultivar. The whole procedure was repeated. Twenty-seven differential results were collected for each isolate in one round of experiments, and data were recorded.

A differential cultivar is defined as susceptible if more than 60% of its seedlings were killed and resistant if less than 40% of its seedlings were killed. In this experiment, if more than three or more seedlings per pot were killed, the isolate was considered as being able to defeat that cultivar; if two or fewer seedlings per pot were killed, the cultivar was considered resistance to this isolate. Wounds on seedlings became dark brown to black, shrank, and dried after the plants were exposed to the light. Infected seedlings were yellow, wilted, and dried because of no nutrients and eventually died. Isolates were classified into races based on their reactions on the eight resistant alleles used in the differentiation tests.

Results

Plant samples

There were 19 isolates recovered from plant samples from 22 fields in 10 counties (Buchanan, Greene, Jasper, Johnson, Lee, Marion, Marshall, Polk, Ringgold, and Wayne). The results of race test of isolates from plant samples with the virulence formula of the races are shown in Table 2.1. Eighty percent of the isolates from plant samples were classified into races, and 20% of the isolates did not fall in to any published race category (Tables 2.2 and 2.3). The percentage of isolates virulent to *Rps* 1k was 73.3%, and the percentage of isolate virulence to *Rps* 7, *Rps* 1c, and *Rps* 1a was 86.7, 46.7, and 73.3%, respectively. Three new races that were not present in the Iowa field during the last survey were identified in this test. They were races 20 (vir. *Rps* 1a, 1b, 1c, 1k, 3a, 7), 28 (vir. *Rps* 1a, 1b, 1k, 5, 7), and 35 (vir. *Rps* 1a, 1b, 1c, 1d, 1k, 7) (Tables 2.3 and 2.4). All these new races could overcome the *Rps* 1a, 1k, and seven genes. Race 28, 15.8% of the total isolates from plants, replaced race 3 in becoming the most dominant race found on plant samples. Race 1 (vir. *Rps* 7) was the second most dominant race in this study as well as in the previous survey.

Three isolates were found with unpublished virulence formulas (Sully Coop 1-3: *Rps* 1c; Polk 3-3: *Rps* 1b, 1d, 3a, 6; Marion 1-2: *Rps* 1a, 1b, 1c, 1d, 1k, 3a, 7) and did not fall into any race category. Among the unidentified isolates, Sully Coop 1-3 (*Rps* 1c) could be race 18, which is a new race that was first found in Iowa, or it could be another new race, which

could only defeat *Rps* 1c gene. Because the other published races that could overcome *Rps* 1c also could defeat other genes such as *Rps* 7 or *Rps* 1a, except race 18 (*Rps* 1c, 3b). However, because no differential cultivar with *Rps* 3b gene was used in the race test, this isolate could not be classified to race 18. Isolate Marion 1-2 defeated seven of eight resistance alleles used in the test. This isolate also had the fastest vegetative growth rate among all the isolates tested in the next chapter. Polk 3-3 defeated *Rps* 3a gene, which was reported resistant to 77% of the isolates recovered from Michgan (Kaitany *et al.*, 2001) and 73.3% of the isolates recovered from plant samples in this survey.

Race 25 was first found on soybean plants in Iowa (Yang *et al.*, 1996) in 1994, but only 1% of the isolates at the time were classified as race 25 (vir. *Rps* 1a, 1b, 1c, 1k, 7). However, race 25 became the other most dominant race on soybean plants with the same percentage as race 28 in the 2001 survey. Among all the 15 tested isolates, 11 could overcome *Rps* 1k. This percentage was as high as 73.3%.

Soil samples

In total, 36 isolates were isolated from soil samples, and 85.3% of the available isolates were classified to race. Race test results and virulence formula of the isolates are shown in Table 2.5. Isolates that could defeat the *Rps* 1k gene made up 35.3% of the total soil sample isolates. The chance of overcoming *Rps* 7 was 85.3%, suggesting that this allele had lost its effectiveness. A similar trend applied to *Rps* 1a, which had a 61.8% chance of being defeated. Among the five unclassified isolates, four could overcome the *Rps* 1k gene and two have the same virulence formula (NC-9 and SC-7) (*Rps* 1a, 1c, 1d) (Table 2.6). Races 1 and 3 were the dominant races found from soil samples, with the same detection percentage (17.6%).

Race 1 was less prevalent than race 3 in the previous survey but more prevalent than race 3 in 1994 study. However, it achieved the same prevalence as race 3 in the 2001 assessment. Race 2, which was 5.8% of the isolates from soil samples, was not recovered during the 1991 to 1994 survey on soil samples, but made up 4.6% of recovery in plant samples in 1991. Race 13 was similar to race 2, only it was recovered from this survey at 8.8%. Races 15 and 20 had not been recovered from soil samples yet, but they were isolated from diseased plants. Race 15 was isolated with 1% frequency in 1994 and race 20 with a 13.3% frequency in 2001 and 2002 (Table 2.4). These differences suggested that the diversity of *P. sojae* races in the field could be much higher than what we detected because

some of the races might not be compatible with the cultivars used in the fields, but they could still survive in the soil undetected due to the relatively small amount of the samples. Two new isolates that had not been reported before this survey were found, races 28 and 35, at 2.9 and 11.8%, respectively. Races 3 and 4 kept decreasing their percentage among the isolates from 29.3% in 1992–1993 to 20% in 1994 to 17.6% in 2001–2002, 31.4% in 1992–1993 and 20% in 1994 to 5.8% in 2001–2002, respectively.

Five isolates with four virulence formula that had not been reported before were identified (NC-2A: *Rps* 1a, 1c, 1d; NC-9 and SC-7: *Rps* 1a, 1b, 1c, 1d, 1k, 6, 7; NW-5A: *Rps* 1a, 1b, 1c, 1k; CE-10: *Rps* 1a, 1b, 1d, 1k, 6, 7) (Tables 2.4 and 2.6). All of them defeated *Rps* 1a gene and four of five of these unidentified races could overcome the *Rps* 1k gene.

Discussion

A population shift in *P. sojae* races in Iowa was detected in this survey, both from plant samples and soil samples, when compared with the previous survey (Table 2.4). Races that were virulent to *Rps* 1k became more common, with 5% in the previous survey and 45% in this survey. There were several races presented both on plant samples and in soil samples previously, but they were undetectable on plant samples, such as race 3. The frequency of occurrence of races of *P. sojae* differed from that in plant samples and soil samples. Races with numbers lower than 15, including race 15, which contained no virulence gene to *Rps* 1k had higher isolation frequency in soil than on plants. Races with the *Rps* 1k virulence gene (with race number equal or higher than 20) all had higher frequency of occurrence on plants than in soil except race 35.

Compared with the previous 1991 and 1994 assessment (Yang *et al.*, 1996), population of races on plants that could not defeat *Rps* 1k gene were declining, whereas the population of races that were virulent to the gene significantly increased ($P < 0.05$) according to chi-square test (Table 2.2). Only 4.6% of the isolates from plant samples could defeat *Rps* 1k in the 1991 study, and 1% in 1994, whereas 73.3% of the plant sample-derived isolates had the gene virulent to *Rps* 1k. Races that were prevalent during the last survey such as race 3 (vir. *Rps* 1a, 7) and race 4 (vir. *Rps* 1a, 1c, 7), with 32 and 18%, respectively, were no longer detected in this study. Races 2 (vir. *Rps* 1b, 7), which was only 4.6% of the plant-isolated isolates in 1991 and was not detected in 1994; 8 (vir. *Rps* 1a, 1d, 6, 7), which was only detected at 0.98% in 1994; 13 (vir. *Rps* 6, 7), which was only recovered in 1994 at 2%; and 15 (vir. *Rps* 3a, 7), which made up 1% of the sample

population in the previous assessment, were all undetectable. Races 20, 25, 28, and 35 could defeat *Rps* 1k, and they became more popular. This trend is shown in Fig. 2.2.

As with plant sample isolates, chi-square test showed that soil sample isolates experienced a significantly increase of *Rps* 1k avirulence gene as well as *Rps* 1b, 1d, and 6, in the pathogen population ($P < 0.05$) (Table 2.5). Defeat frequencies of *Rps* 1b, 1c, 1d, and 3a also increased significantly from the results of this survey compared with the previous survey ($P < 0.05$) (Table 2.5). These shifts indicated the lost of effectiveness of these *Rps* genes in the fields. Races do not have *Rps* 1k virulence gene such as races 3 and 4 kept decreasing their percentage among the isolates from 29.3% in 1992–1993 and 20% in 1994 to 17.6% in 2001–2002, 31.4% in 1992–1993, and 20% in 1994 to 5.8% in 2001–2002. Two new races, 28 and 35, both could defeat *Rps* 1k, whereas another race, race 25, which also could overcome the gene, expanded its population compared with the previous survey. Races that were not virulent to *Rps* 1k showed decreasing trend, but most of them were still detectable from the soil samples, except race 5 (Fig. 2.4). Despite the change of other races, race 1 was still of the highest proportion among all the isolates from soil. Even with the selective pressure, race 1 was still abundant in the field population, whereas race 3 was undetectable in the fields.

Change in *P. sojae* population structure is not uncommon. The population structure change of *P. sojae* at each location had a common trend: the increase of virulence complexity of the phenotypes (races). Before 1975, only race 1 had been reported in Iowa (Tachibana *et al.*, 1975), but the diversity of *P. sojae* races seems to have increased. The dominant races in Iowa were similar to those in neighboring states. Illinois, Indiana, Minnesota, and Ohio as well as Iowa all reported race 1 as the first race of *P. sojae* in these states (Laviolette *et al.*, 1983; Schimitthenner, 1989; Leitz *et al.*, 2000). In Ohio, race 1 had the highest isolation frequency, 20.8%, from plant samples during survey conducted in 1978 to 80 by Schimittthnner *et al.* (1994b) but was not recovered from any of the plant samples in 1990. From 1973 to 1990, races 1, 3, 4, 5, 7, 8, and 9 were reported in Indiana and Ohio additional to the previous identified races such as races 1 and 3 (Schimittthnner *et al.*, 1994b). These two states have the most diverse population of *P. sojae* because they have the longest history of PRR occurrence. Similar complexity increase had been observed in Iowa and Michigan as well as in Ontario, Canada. Races 2, 3, 4, 5, 13, 15, and 25 were recovered in 1994 additional to race 1 in Iowa (Yang *et al.*, 1996), whereas in Michigan,

races 1, 3, 4, and 6 were the only ones present in fields before 1976 (Lockwood *et al.*, 1978), but races 2, 25, 41, and 44 were isolated in Michigan in a recent survey (Kaitany *et al.*, 2001). In Ontario, races 3, 4, 5, and 6 were identified during 1973 to 1976, and races 7 and 9 were found in addition to the previous results in 1979. Between 1980 and 1989, races 8, 13, and 21 occurred in fields with the other known races (Anderson *et al.*, 1992). During 1979 to 1996, eight new races, 46 to 53, were identified in addition to 14 known races in Australia, compared with the previous study (Ryley *et al.*, 1998).

It was reported that approximately 20% of the isolates recovered from Michigan had 10 or more virulence genes corresponding to *Rps* genes (Kaitany *et al.*, 2001). In Iowa, 44.9% of the isolates tested in 2001–2002 showed virulence to five or more *Rps* genes. Four of eight isolates without assigned race numbers (potential new races) contained virulence genes corresponding to more than five *Rps* genes. This fact could lead to two potential problems that need to be addressed. The first is to preserve the simple races required for breeding and screening programs, and the second is the limited potential of recycling deployed *Rps* genes (Dorrance *et al.*, 2003).

In Iowa, counties that had *P. sojae* with the ability to defeat *Rps* 1k gene inlcuded Bucanan, Dickinson, Fremont, Greene, Jasper, Johnson, Lee, Madison, Marion, Marshall, McNay, Sioux, Ringgold, and Wayne (Table 2.6; Figs. 2.3 and 2.4). *Rps* 1k virulent races infected southern Iowa more severely than northern Iowa (Fig. 2.4). Previous research indicated that several southwestern Iowa counties had *P. sojae* races with avirulence gene of *Rps* 1k. However, only one county in southwestern Iowa was confirmed to have these *P. sojae* races in this study.

There are several possible reasons causing the population shift. In agricultural environment, human activities always have great impact on disease epidemiology. The cultivars planted in the fields and management strategies affect the population dynamics of pathogens. Selection pressure, application of single gene varieties should be the number 1 reason contributing to this population shift. Before 1994, PRR was known to occur on Iowa soybean cultivars with the *Rps* 1 and the *Rps* 1c resistant alleles. So, new cultivars with *Rps* 1k were developed and widely planted in recent years. However, this increasing selection pressure could suppress the races without the avirulence gene corresponding to *Rps* 1k and enable virulent races to expand their population and became dominant. Population shifts in other states also were noted with the recent large-scale planting of certain cultivars (Anderson *et al.*, 1992; Dorrance *et al.*, 2003). It had been predicted that single allelic

resistance did not offer long-term protection against the pathogen. Along with the problem of an increase in complexity of virulence genes in races, it is necessary to use new genes for resistance to PRR.

Because all the races in the *P. sojae* population should have received an equal amount of selective pressure, race 1 could always be detected in all the soybean fields that were sampled, whereas race 3, the same as race 1, without virulence to *Rps* 1k, decreases in frequency of being isolated. Having weaker fitness components could be one of the reasons for the shift. Races with weaker fitness characters will lack nutrient resources in competition with more aggressive races.

Pathogens themselves will have random changes within the population, and the new phenotypes (races) eventually can establish if they have competitive fitness characters over other phenotypes (races). Accidental crossing is one of the mechanisms that generate new phenotypes (races). *P. sojae* is homozygous, but occasionally it can outcross and bring new genes into the population (Bhat *et al.*, 1993). Races with these new genes could become prevalent if environmental conditions are favorable and could defeat the popular resistance alleles. Drenth *et al.* (1996) explained the low level of genotype diversity of *P. sojae* population in Australia by indicating a low percentage of outcrossing in vitro for *P. sojae* from one single introduction. Förster *et al.* (1994), Whisson *et al.* (1994), Henry *et al.* (1995), and Chamnanpunt *et al.* (2001) also performed similar studies that explained that the high level of genetic variation of *P. sojae* in the United States was because of low outcrossing within the population. The increase of the virulence genes in races was the consequence of the outcross as well.

Mutation is another pathway for diversification of the *P. sojae* races. Mutated genes that lead to proper fitness characters to the natural and cultured environment would make these races be more competitive compared with others (Förster *et al.*, 1994; Drenth *et al.*, 1996). Because of mutation, *P. sojae* isolates can easily lose pathogenicity. The eight isolates with new virulence formulas could not be classified as new races in the unrepeatable test results, such as Sully Coop1-3 and Polk 3-3. Molecular techniques may be helpful in determining the genetic difference between the eight isolates and the existing races.

Searching for more new resistance genes and incorporating more *Rps* genes into soybean cultivars with good yield potential would be one effective way to control PRR. In Indiana, *Rps* gene combination (1k or 1c +3a or 6) will be effective to control 95–98% of the

races identified from the fields (Cochran *et al.*, 1999). Because the races of the pathogens involve more virulence genes within the new races, breeding new cultivars with several *Rps* could increase the partial resistance.

Although there have been no severe outbreaks of PRR in recent years, because 82 of 168 PRR-resistant cultivars/brands used in Iowa had *Rps* 1k gene and a larger population of races that can defeat this gene has occurred in the Iowa soybean fields, the outbreak of this disease should be expected in any year when frequent rainfalls occur associated with warmer temperatures. Closer monitoring of the population and changes in resistance genes in commercial cultivars should be implemented.

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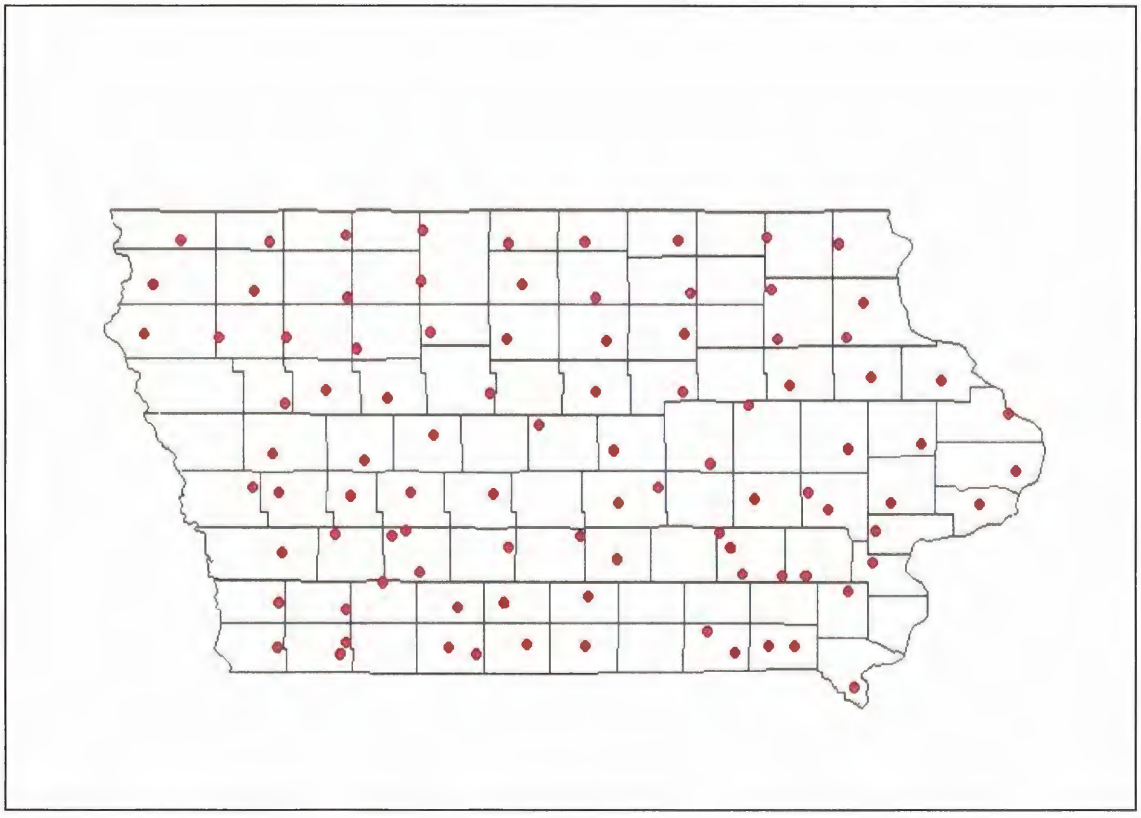

Fig. 2.1. Soil sample map of *P. sojae* in 2001. The red spots show location of the sample fields.

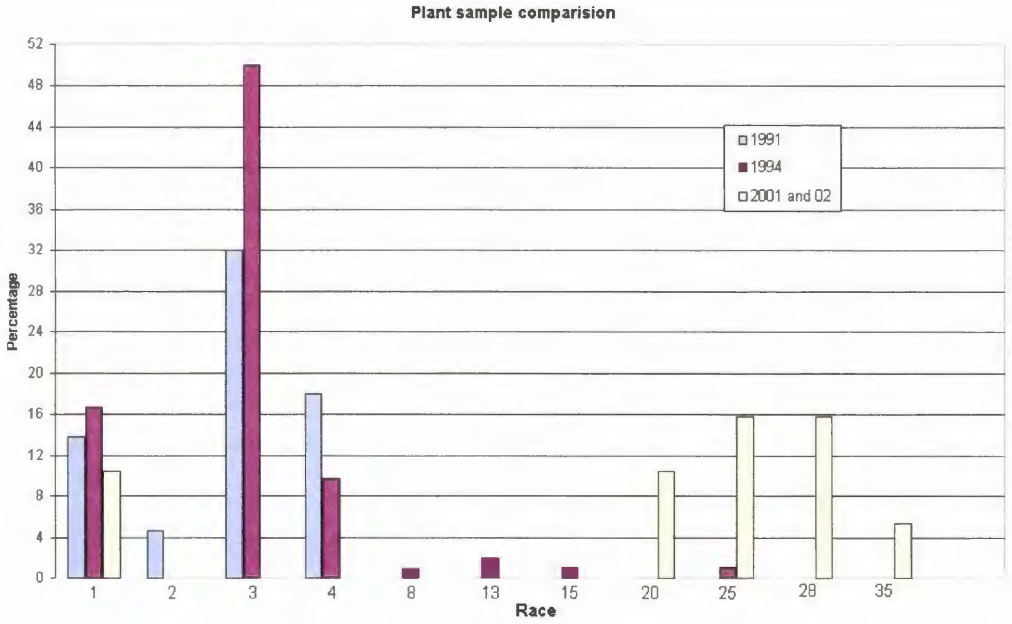

Fig. 2.2. Comparison of *P. sojae* race frequency between 1991 and 1994 and 2001 and 2002 surveys for samples from soybean plants in Iowa.

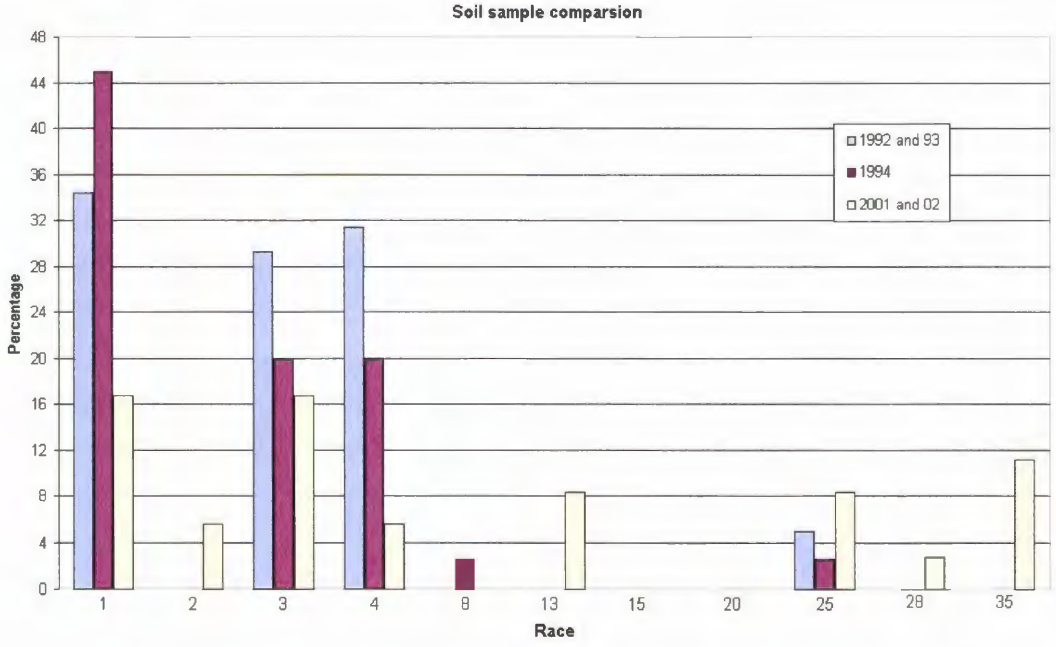

Fig. 2.3. Comparison of *P. sojae* race frequency between 1991 and 1994 and 2001 and 2002 surveys for soil samples from soybean fields in Iowa.

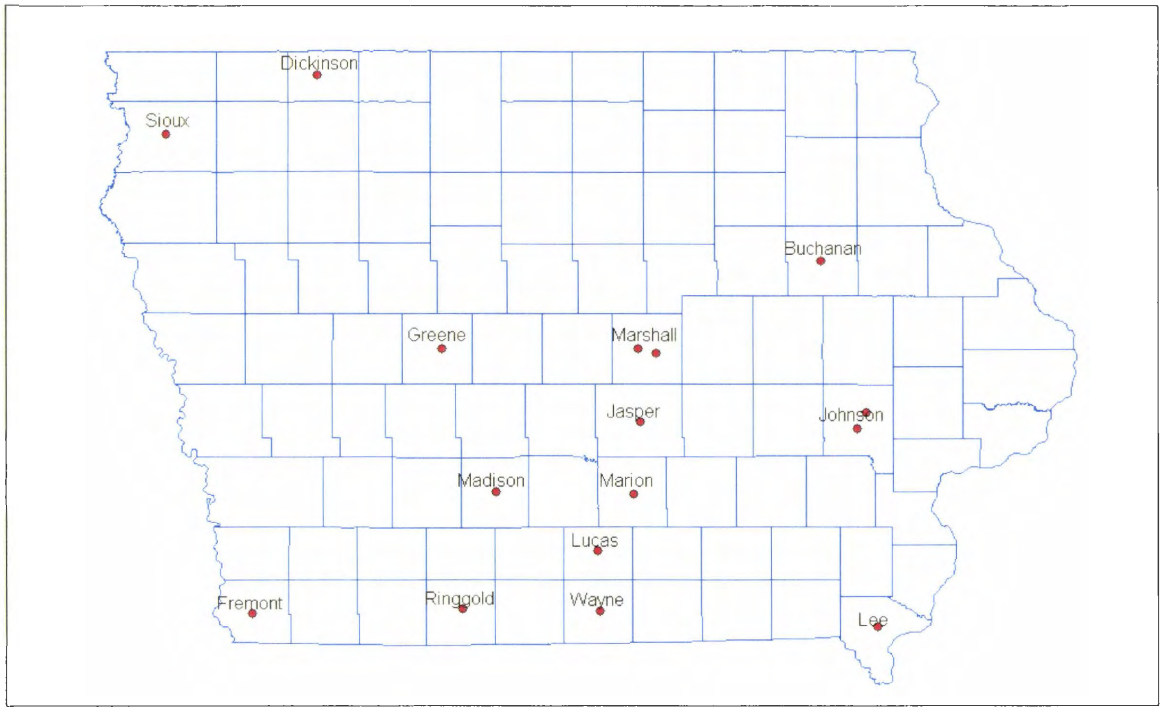

Fig. 2.4. Iowa counties with fields infested by races of *P. sojae* that can overcome *Rps 1-k* gene of soybean.

Table 2.1. Differential varieties with different *Rps* genes used in the 1991–1994 survey and this 2001–2002 survey

Gene[a]	Cultivar	Cultivar	Cultivar[b]
	1991–1993	1994	2001–2002
Rps 1-a	BSR-101	L75-61	L75-61
Rps 1-b	Sanga	L-77	Sanga
Rps 1-c	Mack	Harosoy	Harosoy
Rps 1-d	PI 103.091	PI 103.091	PI 103.091
Rps 1-K	Kingwa/PI103	Williams 82	Williams 82
Rps 3	PI 171.442	Harosoy	Harosoy
Rps 6	Altona	Altona	Altona
Rps 7	Harosoy63	Harosoy63	Harosoy63
rps	Williams	Williams	P9344

[a] rps is susceptible gene and used as control in the experiment.

[b] P9344 is a cultivar produced by Pioneer Hi-Bred International without any resistant gene.

Table 2.2. Comparison of plant derived *P. sojae* isolates collected in 1991–1994 survey and 2002 survey for changes in the number of isolates that had susceptible interactions with the *Rps* genes

% of isolates	1991 plant samples	1994 plant samples	2001–2002 plant samples
Rps 1a	54.5	61.8	73.3
Rps 1b	4.6	1	80*
Rps 1c	22.7	10.8	46.7*
Rps 1d	4.6	0.98	20*
Rps 1k	4.5	0.98	73.3*
Rps 3a	0	1.96	26.7*
Rps 6	0	2.9	6.7
Rps 7	100	100	86.7

* Values are significantly different from the ones from previous two studies with chi-square test at 5% significance.

Table 2.3. *P. sojae* race test results of plant sample derived isolates and virulent formula of the isolates

Samples from plants		
Isolate[a]	Race[b, c]	Virulence formula
Polk 3-3	N/A	*Rps* 1b, 1d, 3a, 6
Polk 3-6	1	*Rps* 7
Marshall2-4	28	*Rps* 1a, 1b, 1k, 5, 7
Marshall2-1	25	*Rps* 1a, 1b, 1c, 1k, 7
Marshall1-2	28	Rps 1a, 1b, 1k, 5, 7
Marshall1-1	25	*Rps* 1a, 1b, 1c, 1k, 7
Jasper5-3	28	*Rps* 1a, 1b, 1k, 5, 7
Greene2-2	1	*Rps* 7
Lee1-1	20	*Rps* 1a, 1b, 1c, 1k, 3a, 7
Marion 1-2	N/A	*Rps* 1a, 1b, 1c, 1d, 1k, 3a, 7
Sully Coop1-3	N/A	*Rps* 1c
Sully Coop3-2	20	*Rps* 1a, 1b, 1c, 1k, 3a, 7
Sully Coop5	35	*Rps* 1a, 1b, 1c, 1d, 1k, 7
McNay 1-3	25	*Rps* 1a, 1b, 1c, 1k, 7
Buchanan15	41	*Rps* 1a, 1b, 1d, 1k, 7

[a] Isolates from plant samples were named after the counties that the samples came from. The first number in the isolate name represents the number of the sample used to obtain the isolate, and the second number represents the number of the isolate used to perform the test.

[b] Numbers in the race column are the races of the isolates. N/A, isolate was not identified into races because of no published virulent formula is available. They could be new races.

[c] An X in race column means the isolates cannot be purified.

Table 2.4. Percentage of races of *P. sojae* tested in 2001–2002 compared with those in the survey in 1991–1994

	1991	1992 and 93	1994	1994	2001 and 02	
Race [c]	Plants	Soil	Plants	Soil	Plants	Soil
1	13.8	34.4	16.7	45	13.3	17.6
2	4.6	0	0	0	0	5.8
3	32	29.3	50	20	0	17.6
4	18	31.4	9.8	20	0	5.8
5	0	0	0.98	2.5	0	0
13	0	0	2	0	0	8.8
15	0	0	1	0	0	0
20	0	0	0	0	13.3	0
25	0	5	1	2.5	20	8.8
28	0	0	0	0	20	2.9
35	0	0	0	0	6.7	11.8
Total Isolates Tested	22	42	102	40	15	34

[c] New races are in red.

Table 2.5. Comparison of soil-derived *P. sojae* isolates collected in 1991–1994 survey and 2002 survey for changes in the number of isolates that had susceptible interactions with the *Rps* genes

% of isolates	1992–1993 soil samples	1994 soil samples	2001–2002 soil samples
Rps 1a	64.3	45	61.8
Rps 1b	4.8	1	41.2*
Rps 1c	35.7	22.5	35.3
Rps 1d	0	2.5	23.5*
Rps 1k	5	2.5	35.3*
Rps 3a	0	0	0
Rps 6	0	2.5	20.6*
Rps 7	100	100	85.3

*Values are significantly different from the ones from previous two studies with chi-square test at 5% of significance.

Table 2.6. *P. sojae* race test results for soil sample derived isolates and virulent formula of the isolates

Isolates from soil			
Isolate[a]	Race[b, c]	Virulence formula	Sample location (county)
NC-2A	N/A	*Rps 1a, 1c, 1d*	Cerro Gordo
NC-8	35	*Rps 1a, 1b, 1c, 1d, 1k, 7*	Winnebago
NC-9	N/A	*Rps 1a, 1b, 1c, 1d, 1k, 6, 7*	Worth
NE-3A	1	*Rps 7*	Fayette
NW-1	3	*Rps 1a, 7*	Pocahontas
NW-4	3	*Rps 1a, 7*	Plymouth
NW-5A	N/A	*Rps 1a, 1b, 1c, 1k*	Sioux
NW-5D	13	*Rps 6, 7*	Sioux
NW-8A	3	*Rps 1a, 7*	Osceola
NW-8B	4	*Rps 1a, 1c, 7*	Osceola
NW-9B	25	*Rps 1a, 1b, 1c, 1k, 7*	Dickinson
NW-10	3	*Rps 1a, 7*	Osceola
CC-5A	1	*Rps 7*	Grundy
CC-5B	3	*Rps 1a, 7*	Grundy
CC-5C	23	*Rps 1a, 1b, 6, 7*	Grundy
CC-8	1	*Rps 7*	Hardin
CC-10D	2	*Rps 1b, 7*	Dallas
CE-5	1	*Rps 7*	Jones
CE-10	N/A	*Rps 1a, 1b, 1d, 1k, 6, 7*	Johnson
CE-20	25	*Rps 1a, 1b, 1c, 1k, 7*	X
CW-1	25	*Rps 1a, 1b, 1c, 1k, 7*	Greene
SC-1A	28	*Rps 1a, 1b, 1k, 5, 7*	Madison
SC-4A	35	*Rps 1a, 1b, 1c, 1d, 1k, 7*	Ringgold
SC-7	N/A	*Rps 1a, 1b, 1c, 1d, 1k, 6, 7*	Wayne
SC-8A	13	*Rps 6, 7*	Lucas
SC-10	1	*Rps 7*	Marion
SE-10A	35	*Rps 1a, 1b, 1c, 1d, 1k, 7*	Johnson
SW-1	3	*Rps 1a, 7*	Adair
SW-6	35	*Rps 1a, 1b, 1c, 1d, 1k, 7*	Fremont
SW-11A	1	*Rps 7*	Adair
SW-11B	13	*Rps 6, 7*	Adair

[a] Isolates were named by the location that they were collected. N, north; W, west; S, south; and E, east. Numbers in names of isolates represented the order that the sample was collect and the letters followed the numbers mean in which leaf disc that the isolates were isolated from, for example, NC-2A means the isolate was collected in North Central Region, and it was the second sample collected in that location and it was isolated from leaf disc A.

[b] Numbers in the race column are the races of the isolates. N/A, isolate was not identified into races because of no published virulent formula available.

[c] An X in race column means the isolates could not be purified.

FITNESS COMPONENT STUDY OF *PHYTOPHTHORA SOJAE* RACES

Abstract

Studies were conducted to compare fitness parameters of *Phytophthora sojae* races isolated from Iowa soybean fields to explain and predict population shift of *P. sojae.* Single-zoospore isolates of five races were cultured on V-8 agar at 15, 20, 25, and 30°C, and fungal growth rates were compared by measuring colony diameters daily for 5 d. After 6 d, eight 1-cm^2 plugs from each colony were cultured using salt washing method for zoospore production and quantification. Trials and tests were performed for testing mycelial growth rates of difference races of *P. sojae*, the zoospore productivity, and infection aggressiveness of different races. Infection aggressiveness of *P. sojae* was determined by the virulence index value (VIV), which was reciprocal of host resistance index (HRI), and calculated by formula VIV = $1+(ab^{-1})$, where *a* is disease incidence (%) and *b* is latent period (hours/days). Leaf disc dipping inoculation method, which involved dipping sterilized leaf discs in zoospore suspension for 1.5 and 15 min for the infection to occur, was adapted in the experiment. Significant differences among races were observed for colony diameter, zoospore production, and infection aggressiveness; differences could help to explain the race shift and may even predict it. Zoospore production rates (number/ml) in order of races were 35 > 1 > 3 > 25 > 28; VIV of races was in the order of races 25 > 35 > 1 > 3 > 28. Race 1 grew fastest at all four temperature treatments. Together with its medium ability of spore production and infection aggressiveness, race 1 might maintain or lose its popularity in the future. Races 25 and 35 were more aggressive when infection occurred compared with the other three tested races, and with their medium-to-high capacity of vegetative development and spore production, they might become dominant races in Iowa soybean fields. Races 3 and 28 had the weakest fitness parameters and might further decrease their proportion in the population. Additional study is needed to expand the knowledge of fitness characters of races of *P. sojae* and to predict the population shift based on the information.

Introduction

Phytophthora root rot (PRR) is one of the most devastating soybean diseases in the United States. It causes >1,000 thousand metric tons of yield loss on soybean, *Glycine max*,

annually in the United States (Wrather *et al.*, 2001). Oomycete *Phytophthora sojae* M.J. Kaufmann & J.W. Gerdemann (Syn. *P. megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin), is the causal agent of this disease. Currently, >70 races (phenotypes) of *P. sojae* have been defined, based on their virulent formula on eight or 13 differential cultivars (Dorrance *et al.*, 2003).

From 1948, the year PRR was first noted, until the 1990s, race 1 was the one with the highest isolation frequency in most of the states that claimed PPR occurrence (Schmitthenner, 1989; Yang *et al.*, 1996; Leitz *et al.*, 2000; Giesler *et al.*, 2002; Dorrance *et al.*, 2003), regardless of the widely used *Rps* 1k gene, which has resistance to race 1. However, race 3, prevalent in Iowa, also cannot defeat the *Rps* 1k gene (Yang *et al.*, 1996) and is not as frequently detected as previously. Goodwin (1998) indicated that analysis of fitness components was a highly promising area of research, because it could help answer questions about the population replacements that were probably a common aspect of *Phythophthora* biology. This study was designed to quantify the fitness characters of some races of *P. sojae* and to determine whether there are any correlations between population changes of a certain race and its fitness components.

Fitness was defined as the contribution made by a genotype to the gene pool of the next generation (Tooley *et al.*, 1986). The most important fitness components are usually described as latent period, infection efficiency, asexual reproduction, overwintering ability, and lesion size (Tooley *et al.*, 1986; Goodwin, 1998). For *P. sojae*, zoospore production, mycelial growth, and aggressiveness are the likely parameters contributing to the fitness of different races. Aggressiveness of some race was valued by latent period, infection efficiency, and the reciprocal of host resistance index (HRI) (Thakur *et al.*, 1997), where *a* is infection incidence (%) and *b* is latent period (hours).

Oospores are the primary inoculum source for *P. sojae* (Athow, 1985). However, zoospores are the main infective unites (Vedenyapina *et al.*, 1996). Zoospores can be transmitted by splashing rain and are the source of secondary infection of this disease. A significant role of zoospores is in dissemination, which involves not only production and movement of inoculum to a host plant but also subsequent infection. The ability of producing massive zoospores is one of the most important aggressiveness parameters. To maintain the mortality of zoospores after quantification of the concentration of the spore suspension, a few issues need to be addressed. According to Hickman *et al.* (1966), at temperatures below 10–15°C, *P. sojae* zoospores can survive for 48 h. Hickman *et al.* (1966) also

reported that rapid encystment of *P. sojae* zoospores contributed to frequent contact of zoospores with the bottom of the dish and to contact with glass beads. Zoospores lost motility within 30 min in suspensions approximately 0.03 cm in depth, whereas in suspensions 0.3 cm in depth there was no encystment occurred for hours.

Mycelial growth was used to measure the effect of outside factors on pathogen vegetative development (Vedenyapina *et al.*, 1996). Gijzen *et al.* (1996) did an experiment on temperature-induced susceptibility of soybean with *Rps* genes and found that the susceptibility was generally consistent for specific *Rps* genes, regardless of genetic background. However, no study had been conducted to determine the temperature effect on *P. sojae* mycelial growth.

Detached leaflet and leaf disc assays have been used for assessments of host–pathogen compatibility in *P. sojae* (Abe *et al.*, 2002; Carlisle *et al.*, 2002). Sporangia and zoospores attacking foliage are very common and easily observed (Hickman *et al.*, 1966). Quantitative study of aggressiveness of *P. infestans* has been done in many recent studies in both Europe (Flier *et al.*, 1999; Lebreton *et al.*, 1999) and the United States (Tooley *et al.*, 1986; Legard *et al.*, 1995., Miller *et al.*, 1998). These techniques have been adopted in preference to whole plant assays because they enable the testing of many more isolates simultaneously and allow for tighter control of the environmental conditions for replicate tests without contaminations from airborne pathogens. Tooley *et al.* (1986) compared the fitness components between sexual and asexual population of *Phytophthora infestans* (isolates from Mexico and United States) and found significant differences among isolates in infection frequency, lesion size, sporulation capacity, and composite fitness index. Thakur *et al.* (1997) used HIR = $[1 + (ab^{-1})]^{-1}$, where a is infection incidence and b is latent period, to measure the resistance level of the host plants. The more resistant the hosts, the higher the HIR value. In contrast, pathogens that cause more severe symptoms on hosts that have same HIR value were more virulent.

Studies also have been conducted on testing single-isolate aggressiveness or mycelial growth of *P. sojae*, but no systematic study has been conducted to determine the difference in fitness components among races. This study was designed to quantify the aggressiveness, temperature effects on the vegetative growth, and the asexual spore production of races of *P. sojae*.

Materials and Methods

Selected isolates

Races 1, 3, 25, 28, and 35 were selected based on our interest in their population shifts (see Chapter 2). Between the two previously dominant races 1 and 3, race 1 still maintains high frequencies of isolation from soil and plant samples, whereas no race 3 was recovered from the 2001–2002 survey. To compare whether fitness characters played roles in these shifts, four isolates of race 1 (NE-3A, CC-8, CC-5A, and SW-11) and four isolates of race 3 (CC-5B, NW-4, NW-8A, and SW-1), all from soil samples, were randomly assigned for the test. Races 25, 28, and 35 were chosen due to the highly concerned resistance gene, *Rps* 1k. Races 28 and 35 were newly presented races with *Rps* 1k compared with the survey of Yang *et al.* (1996), whereas race 25 showed an increase in percentage in the sample population. Four isolates of race 25 (Marshall1-1, Marshall2-1, CE-20, and CW-1) and two isolates of race 28 (Marshall2-4 and SC-1A) and 35 (SE-10A and SC-4A) were assigned to the test. Uneven numbers of isolates were chosen because of an insufficient number of race 28, whereas only the two selected isolates of race 35 were able to steadily produce zoospores in our preliminary studies.

Mycelium growth rate on medium

Growth media included dilute V-8 juice medium, water agar, and oatmeal agar, all of which are widely used in *P. sojae* studies. In a preliminary experiment, dilute V-8 juice medium (Schmitthenner *et al.*, 1994) was determined to be the most suitable medium in fitness character testing, not only because V-8 medium supports *P. sojae* at a growth rate easy for measurement (~10 mm growth in diameter under room temperature) but also because *P. sojae* colonies develop in a relatively round pattern, thus measurement is more accurate with regular scale.

In total, five races with 16 isolates were tested. Plates were incubated for 5 d at room temperature, with 10 h of lights-on in the daytime and I14 h of lights-off at nighttime. Zoospores were produced using these plates as described in below under "Production of zoospores by washing plates." Zoospore suspension was diluted until the concentration reached 150-200 spores/ml. Twenty microliters of suspension was pipetted into V-8 juice medium and spread over the plate. The whole piece of agar was inverted in the plates to let the mycelium grow through the agar. Two days later, when single-spore colonies could be seen with unaided eyes, a razor blade was used to cut the colonies off and transfer them to fresh V-8 medium under a dissecting microscope. The plates were incubated in the dark for 6 d. Single-spore plates were then ready to use to guarantee the purity of *P. sojae* isolates

for the following tests, so all isolates used after this step were from the single-spore colonies.

Twelve plugs were transferred from each plate to 12 85-mm-diameter V-8 medium plates by using a #5 cork borer, sealed with Parafilm, and randomly assigned to four temperature treatments. There were 48 plates for each treatment.

Three rounds of preliminary trials with two replications per trial were carried out under room temperature (25°C) to determine whether the races (isolates) had any visible difference in growth rates. Seven isolates, and three races, were tested in the first trial, whereas 16 isolates, and five races, were tested in the other two trials. Three replications, three rounds, were performed in the final experiment. Each isolate had three plates in each round.

Four temperature treatments (15, 20, 25, and 30°C) were established 2 d before the transfer by using incubators maintained in the dark. Thermometers were used to monitor temperature fluctuations, which was in a range of ±1°C around the required temperature. Freshly transferred plates were stored in crispers in a random order, at the middle shelf in the incubators.

Measurements were carried every 24 h. In total, six measurements were recorded. The measurements were taken in the order 15, 20, 25, and 30°C treatments. A 20-cm-long scale was used to measure the parameters of the colonies. If the colonies were not perfectly round, measurements were recorded as the mean of the longest and the shortest parameters. The measurement points were marked and measured at the same location for subsequent measurements.

Zoospore production ability

Sample preparation

Petri dish #25 was selected to culture the pathogen in lima bean extract and to produce zoospores. This dish size was selected because the 2-cm depth and 85-mm parameter provided space to avoid the contacting of zoospores with each other and with the walls of the dishes and also because #25 is the most regularly used size of petri dishes and readily available in stores.

Two plugs of agar with mycelium of each isolate were transferred to freshly made V-8 medium plates from long-term storage tubes. Plates were incubated for 5 d at room temperature, with lights on during the daytime and lights off during the nighttime. Eight 1-

cm^2 agar plugs with mycelium were cut by using a razor blade from the edge of the colonies of each isolates transferred into petri dishes filled with 25 ml of dilute lima bean extract. Plates were incubated in lights for 48 h and then ready to be washed.

Zoospore production

A large beaker and a 50-ml cylinder were prepared for the wash. The dilute lima bean extract was drained (Schmitthenner *et al.*, 1994) from the incubated plates into the beaker, and the agar plugs were kept in the plates. Twenty-five milliliters of Chen and Zentmyer's salt solution (C-Z solution) (Schmitthenner *et al.*, 1994) was poured using the cylinder. The solution was allowed to sit for 15 min and then the old solution was drained from the plates again. The above-mentioned procedure was repeated four times at 15-min intervals. C-Z solution was replaced with sterilized distilled water at the fifth wash. Plates were stored in light without disturbance. Six hours later, the mycelia started forming sporangia, but most of the zoospores were released 10 h after the wash. Two trials were performed before the formal test to see whether these isolates could produce an observable amount of zoospores.

Zoospore concentration

Zoospore suspensions were usually not too dense for direct counting of spores. No special dilution was needed before counting the spores. Magnification was set as 20X, and based on the size of zoospore and the density of the suspension, cells in the four 1/25-mm^2 corners plus the middle square in the central square were counted. The final concentration was calculated following the instructions for use of the hemocytometer.

Infection efficiency study

Make leaf discs

'Sloan' soybean seeds, which have no *Rps* gene, universally susceptible, were used. Seeds were rinsed with 5% bleach to surface sterilize seeds for 30 s and remove possible seedborne fungi. Then, they were rinsed with distilled water until the bleach was removed and planted in a greenhouse.

Four-inch-diameter pots were filled with sterilized peat, perlite, and soil mixture (1:2:1) until 75% full. Ten to 14 seeds were placed on top of the soil, and a 2-cm depth of soil was added above the seeds. The pots were watered until saturated. Pots were then incubated on a greenhouse bench in random order at 28°C with direct lighting provided. Plants were irrigated every day by the greenhouse manager by using tap water.

Approximately 12–20 leaves per pot were harvested. Ten to 15 pots were planted for each replication, depending on the number of isolates used in the tests. Leaves were harvested 18 to 20 d after planting. They were wrapped in moistened paper towels, sealed in zippered bags, and taken back to the laboratory.

Inoculation

Harvested leaves were rinsed in 5% bleach for 30 s for surface sterilization, and the bleach was flushed with sterilized distilled water. Leaves were air-dried on sterilized paper towels. Leaf discs were cut from harvested fresh leaves by using #5 cork borer. Veins were avoided as much as possible.

Two zoospore suspensions were prepared, with concentrations of 10^3 and 10^4. In each suspension, 20 leaf discs were soaked in each zoospore suspension for 1.5 min, and 10 leaf discs were taken out, and another 10 were taken out 15 min after dipping. Leaf discs were flushed in sterilized water and then air-dried. There were four treatments in total, and three replications were conducted for each treatment. The experiment was repeated three times. Leaf discs were then placed on water agar, 10 discs per plate, sealed with Parafilm, and provided with light.

Data collection

Latent period, which was defined as the time period between the leaf discs being inoculated the appearance of disease symptoms, of every isolate was recorded. The number of leaf discs that showed infection symptoms every 24 h was recorded. The number of infected leaf discs in each plate 3 d after inoculation divided by total inoculated leaf discs was recorded as infection incidence (%). No symptom was assigned a value of 0. Aggressiveness of each isolates was valued by the reciprocal of HRI (Thakur *et al.*, 1997), which was calculated by formula $HRI = [1+ (ab^{-1})]^{-1}$, where a is disease incidence (%) and b is latent period (hours/days), the pathogen virulence parameters.

Results

Mycelium growth rate on medium

Mycelium growth rate for preliminary trials

A correlation between increase in diameter of the colonies and time showed a roughly linear relationship (Fig. 3.1), so that slopes of regression lines could be used to compare growth rates. Four isolates from race 1 were the highest with the greatest growth

rates. Two isolates of race 25 had growth rates slower than race 1 and faster than the isolate from race 3.

Eleven isolates from four races were tested in trial 2 as shown in Fig. 3.2A. Again, all the correlations seemed to be linear, except for that of isolate race 35. Three of four isolates from race 1 had the biggest diameters by the end of the test, race 3 isolates had the smallest colony diameters, and two of three isolates from race 25 were in the middle. The slowest growing race 1 isolate still had a higher growth rate than any race 3 isolates, and a similar trend was observed in preliminary trial 1.

All isolates in previous trials were included in the third trial. A total of 16 isolates of the five races were grown into single-spore colonies and measured. As with the first two trials, linear relationships between time and mycelial growth were observed (Fig. 3.2B). Race 1 showed the highest growth rate, whereas race 3 clustered at the bottom of all the lines by the end of the test (Fig. 3.3). Races 25, 28, and 35 were scattered between race 1 and 3, without apparent differences between each other.

F tests showed that *P. sojae* colonies growth linearly over time with r^2 values ranging from 0.97 to 0.99 for all five races tested in trial 3. Thus, a comparison of growth rate of each race could be substituted by comparing the slopes of the growth–time regression line. Race 1 was observed growing significantly faster than all the other races on the medium. Races 35, 25, 28, and 3 had slower growth rates, without significant differences from each other. Races 25 had a lower growth rate interval than race 35, which could grow faster than 25 because of a higher value at the right end of the interval. Colonies of races 28 and 35 developed daily, even though statistically the lowest amount of their growth could be 0 (Table 3.1).

Test for mycelium growth rate

Four temperature treatments were designed, and the data were arranged and analyzed. At 15°C, race 35, represented by gray regression isolates, had significantly larger ($P < 0.05$) colony diameters than all the other races (Fig. 3.3A). Dark blue, red, yellow, and orange regressions, which represented races 1, 25, 3, and 28, respectively, clustered and did not grow significantly faster than each other. Slopes of regression lines were adjusted to intervals with 95% confidence and compared. Intervals that did not have overlap from the others meant that the growth rates they represented were significantly different from the others. Race 1 had a slightly higher rate of growth among four races (0.18–0.24 mm/h), with the similar range as race 25 did (0.16-0.24 mm/h), whereas race 3 and 28 were of lower

growth rates (race 3, 0.17–0.22 mm/h; race 28, 0.17–0.22 mm/h) but insignificantly different from races 1 and 25.

At 20°C, races 1 and 35 had bigger slopes and larger diameters by the end of the measurement (Fig. 3.3B). Race 28 was still with the smallest slopes as in the previous treatments. From the statistical test results, race 1 grew at a significantly higher rate ($P < 0.05$) (0.38–0.44 mm/h) than the other four races. Races 25 and 35 were the second fastest growing races (0.3–0.35 and 0.32–0.35 mm/h, respectively), and race 25 was significantly faster than race 28 ($P < 0.05$), whereas races 3 and 35 were with no such difference from race 28 (0.25–0.30 mm/h).

P. sojae races reacted with a similar pattern at 25°C as at 20°C (Fig. 3.4A). Race 1 regressions again stood on the top of all the regressions with slope range (growth rate) of 0.49 to 0.54 mm/h. Race 35 followed race 1 to be the second fastest growing race (0.39–0.42 mm/h), significantly faster than race 28 (0.36–0.39 mm/h) ($P < 0.05$), and slightly isolated but not significantly different from other regressions. Races 3 and 28 were at the same growth rate level, even though the race 28 growth rate interval was higher overall than that of race 3 (0.31–0.86 mm/h).

Race 1 maintained the fastest growing rate (0.50–0.56 mm/h) at 30°C (Fig. 3.4B), and race 35 (0.49–0.53 mm/h) was second to it, but there was no significant difference between these two races. The other three races, 3, 25, and 28, were all significantly slower than race 1 ($P < 0.05$). Growth rate interval of race 25 (0.45–0.49 mm/h) slightly overlapped with that of race 35, so it was not significantly slower than race 35. However, races 3 and 28 had much slower rates (0.39–0.44 and 0.38–0.44 mm/h, respectively) than race 35, and their regressions are at the bottom of Fig. 3.4B.

Zoospore productivity

Preliminary trials

Two preliminary trials were done before the large experiment without statistical analysis because of the limited amount of samples and to see whether there were easily detectable differences in the capability of producing zoospores among *P. sojae* races. All the isolates tested in the previous experiment were used in the formal, large test. Figure 3.5A and B shows the results from the two preliminary trials. Race 25 had the second highest capacity to produce zoospores, but there could be variation within this race because there were no other samples from race 25 tested. Race 1 seemed to be ranked third for

producing zoospores among the four races tested, but again, the result might not be representative the whole population because of lack of samples. Two isolates from race 3 were tested in preliminary trial 1, and one isolate was tested in preliminary trial 2. They all produced the lowest amount of zoospores compared with other races in the test. These two preliminary trials indicate that some races can produce more zoospore than the others.

Test

Large variation within races 1 and 25 was observed (Fig. 3.6). Isolates from races 3, 28, and 25 were generally consistent. Statistical tests also showed that races 1 and 25 had differences within races ($P < 0.05$) but not within the other three races. After Tukey's studentized range test, races 3 and 25 produced significantly fewer spores than race 35 ($P < 0.05$), whereas no other significant differences were found among other comparisons. Race 1 ranked the second highest in spore production among the five races. Race 3 produced more spores than races 25 and 28.

Infection efficiency

Latent period. Significant differences in latent period were found among both races and isolates ($P < 0.001$) by *t* test (least significant difference, LSD). Race 3 had the longest latent period, and the time of symptom appearance caused by this race seemed significantly slower than for the other races ($P < 0.05$). Isolates from race 35 leaf discs showed symptoms in the shortest time among all the races. The latent period was approximately 7 h shorter than those of race 3 isolates. Race 25 had the shortest latent period followed by race 35, 28, and 1 (Table 3.2), respectively. Even though race 28 had the fourth longest latent period, it still triggered symptoms almost 4 h earlier than that of race 3. Significant differences existed among isolates as well ($P < 0.001$) (Table 3.2). Three of the four isolates of race 3, NW-4(3), NW-8A(3), and CC-5B(3), were among the four isolates that had the longest latent periods. One isolate from race 35 and three isolates from race 25 had the fastest infection rates, ~10 to 11 h. The rest of the isolates scattered in the middle of the distribution and did not have a clear pattern or significant difference between each other.

Infection incidence. Table 3.3 shows infection incidence measured 72 h after the inoculation. In 95% confidence range, the order of the infection incidence (defined as the number of the infected leaf discs divided by the total number of inoculated leaf discs) of these races from high value to low was race 25 > race 1 > race 35 > race 3 > race 28. Race 25 caused the most severe infection on leaf discs; ~73% of the inoculated leaf discs showed

symptoms by 72 h after inoculation. Race 1 infected approximately 64% of the inoculated leaf discs, but it was a nonsignificantly smaller amount than that of race 25. Race 35 was similar, slightly smaller than race 1 isolates. Race 25 infected significantly more leaf discs by the end of the test than did races 3 and 28 ($P < 0.05$), respectively. There was no significant difference within races.

VIV. Because VIV was defined as the reciprocal of HRI, the smaller the HRI, the more virulence the pathogen. Race 25 had the highest value of HRI^{-1}, significantly higher than that of races 28 and 3, respectively ($P < 0.05$), but not significantly different from that of races 1 and 35. Mean HRI^{-1} of race 3 was 0.28 higher than that of race 28. Virulence degree of races was in the order of races 25 > 35 > 1 > 3 > 28 (Table 3.4).

Discussion

This study is the first effort to compare fitness components of *P. sojae* among races under different temperatures. Results of three preliminary trials also showed that under room temperature, there was significant difference ($P < 0.05$) among different *P. sojae* races, and race 1 was always the race that grew the fastest. Although race 3 had a slower growth rate, it was not significantly slower than the other three races. For the four temperature treatments, race 1 had the highest growth rate, except at 15°C, in which it was the second fastest growing race. This fast vegetative growth character might explain why race 1 is still prevalent in Iowa. In susceptible hosts, fast mycelial growth could make race isolates more competitive than isolates of the other races. Races 3 and 28 had the lowest developmental speed at all tested temperatures. Race 35 had the highest growth rate at 15°C and was the second highest in the other three treatments, whereas race 3 was almost always the lowest. Our study on population shift (Chapter 2) showed that although race 35 is a new race, it had a large proportion of all the Iowa isolates tested. In contrast, race 3 population has decreased dramatically in recent years, indicating the possibility of this fitness character to affect the population shift in *P. sojae* races. Furthermore, race 25 grew slower than race 35 under all treatment conditions, whereas race 28 had a similar rate to race 3. Within each race, by comparing the 95% confidence interval, a common trend for growth was that higher temperature (temperature either lower than or equal to 30°C) led to faster growth (Fig. 3.7). Low temperature (15°C) significantly ($P < 0.05$) inhibited the growth of all tested races except race 35. It was reported that the optimum growth temperature of *P. sojae* was ~25–28°C (Schmitthenner *et al.*, 1994), however, from this experiment, races of *P. sojae* grew the

fastest, although not all significantly faster than for the other temperatures for all the tested races, <30°C. If fast mycelial growth is important to population shift, race 28 would not become a major race in the near future because of the slow growth. The decrease of race 3 is mainly due to another factor, the wide use of the *Rps* 1k gene. However, race 28 could defeat this gene, so it might be able to maintain a low population compared with that of race 3 in the near future. Compared with other races, growth rate of race 35 always ranked on the top two, so it might better adapt to the environmental changes. If so, the prevalence of race 35 in Iowa soybean field could be predicted.

Vedenyapina *et al.* (1996) indicated that mycelial growth could affect the spore production rate; thus, the larger amount of vegetative growth could lead to increased asexual spores production. The high amount of zoospore production could be another advantage of the newly prevalent races. Even though the exact concentrations differed at various times, the races that produced large amounts of spores were consistent. Races 35 and 1 were two such races identified from this study. If the spores from these two races germinate and penetrate leaves faster than those of the other races, fitness advantages could explain the population shift, despite of the effect of resistance gene selection pressure.

Zoospores of *P. sojae* were recorded that could survive in water for up to 48 h after release at 10–15°C (Hickman *et al.*, 1966). From our study, they could survive for the same time at room temperature. Zoospores are important inoculum for secondary infection. Mass reproduction of asexual spores enables the fast occupation of infection sites, high chance of penetration, and ability to overcome the other competing races on host resources. Another way to generate sporangia is to flood the culture with sterilized water and incubate them in 4°C for 24 h, and then change the water and store at room temperature until the zoospores are released. This method is less labor-intensive and produces fewer mistakes, because the colonies are attached to the plates and zoospores are washed off. However, a much smaller amount of zoospores is produced using this method compared with the salt solution wash method. There was an observable amount, approximately 20–30, zoospores swimming in one field (10X) under a microscope if the flooding method was applied, whereas the salt solution wash method can trigger hundreds to thousands of zoospores. Because of less generation of zoospores, the flooding method is suitable for production of single-spore colonies. Because large numbers of zoospores were needed in the next experiment, the salt solution wash was the selected method.

Larger amounts of zoospores also can spread by irrigation and spread broader than those of the races that produce fewer spores. According to Workneh *et al.* (1999), PPR starts from the center of the infection point and becomes less severe along the direction of irrigation water. The more zoospores the water carries, the longer the distance they can travel and the more severe disease they cause.

To guarantee infection, inoculum was used 5 h after hundreds of zoospores could be seen under 10X magnification. Because surface-attached spores were flushed off by water, infection should be only caused by penetrated zoospores; therefore, the faster the spores could lose the flagella and germinate growth tubes, the less time they took to initiate infection. However, no visible difference was observed between the infection incidence of unflushed and flushed discs in preliminary trial. Variation did exist among races and isolates. Further study is needed on the temperature effects on zoospore production and zoospore germination rate.

Length of the latent period is directly associated with the time that zoospores needed to germinate and the mycelial growth rate. The shorter the latent period, the faster the infection occurs, which means the quicker the spores shed flagella and germinate. With enough zoospores, races have shorter latent periods could occupy more infection sites that other races without these advantages. Races 25 and 35 were the top two virulence races among the five races tested.

HRI was an index for evaluation of host resistance (Thakur *et al.*, 1997). The greater the $1 + (ab^{-1})$, the smaller the HRI. The smaller the HRI, the more susceptible the hosts and the more virulent the pathogens. On the contrary, the higher the pathogen virulence index (the reciprocal of HRI), the more virulent the pathogens. Since races that had shorter latent period had higher VIV as well, latent period could be the one of the factors that affected the final VIV the most.

Since all the three components tested in this experiments corresponded with the observed population shifts of races of *P. sojae*, one or all of these components could be used to explain the shifts, although they were not the only reasons. From all the parameters in this experiment, the dominance of race 35 could be attributed to its fastest mycelial growth rate at 15°C and second fastest rate at the other three temperature treatments, and to its highest spore production ability, and second highest virulent index value, whereas race 25 had the highest virulent index value with third fastest growth rate and spore production amount also lead to its high frequency of occurrence. Plus, with their *Rps* 1k virulence gene,

the shift of the races 25 and 35 population in Iowa soybean fields could be explained and predicted. It is likely that these two races will become the dominant races in Iowa soybean fields in the near future. Even though race 1 does not contain the *Rps* 1k virulence gene, its mycelial growth was the fastest among the five races at 20, 25, and 30°C, and it produced the second highest amount of zoospores. Its virulence index value was in the middle of these five tested races. Due to the large accumulated amount of population in Iowa soybean field over ~40 yr and the fitness advantages, race 1 might be able to keep itself in the population at a certain level in the Iowa soybean fields in the short future. However, because of lacking of ability to attack soybean plants with *Rps* 1k gene and the weaker fitness characters comparing to races 25 and 35, race 1 might eventually lose the dominant status in *P. sojae* population in Iowa. Race 3 in every aspect had the second weakest fitness characters, neither it is virulent to the *Rps* 1k gene. Its population could be suppressed by other competitive races such as 35 and 25 and decrease. Race 28 had the weakest fitness advantages despite having *Rps* 1k virulence gene. Although it had a high detected percentage, with the noncompetitive fitness characters detected in this study, it might not be able to survive and expand its population in the long term.

With further study, the information generated from our study should be useful for explaining and predicting the population shifts of race population of *P. sojae*. More races need to be tested to complement the information on fitness characters of races of *P. sojae*. More isolates within races from different locations also need to be tested to see whether greater variation exists among the isolates.

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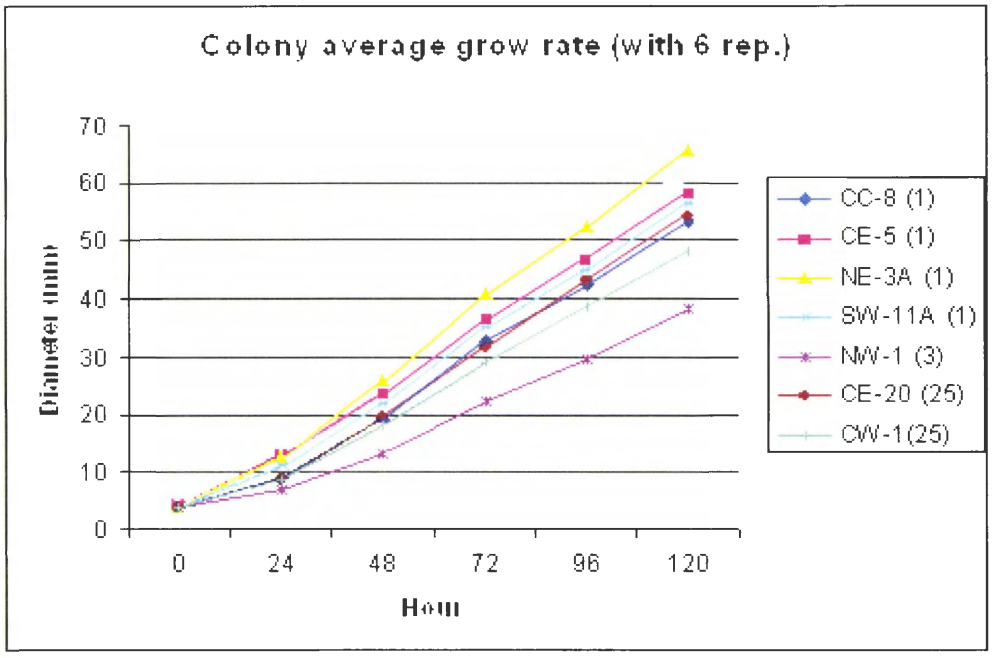

Fig. 3.1. Preliminary trial 1, testing mycelial growth of *P. sojae* over time in V-8 juice medium at room temperature. The experiment was terminated when the first colony reached the edge of the plate.

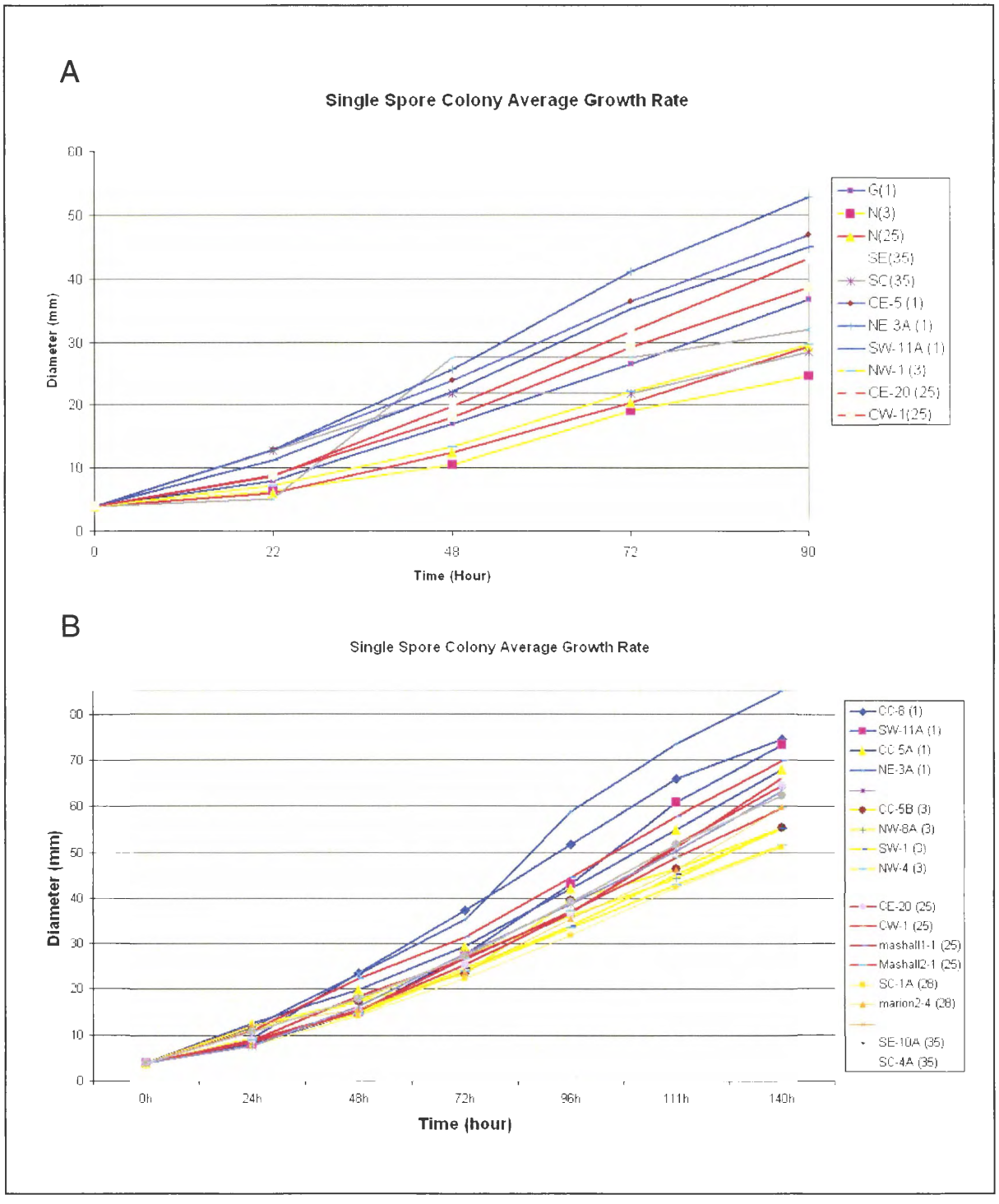

Fig. 3.2. Preliminary trials, testing mycelial growth of *P. sojae* over time in V-8 juice medium at room temperature. Both of the trials used single spore colonies. (A) Results from trial 2, which ended 5 d after inoculation. (B) Results from trial 3, in which the measurement was ended when the fastest growing colony reached the edge of a plate.

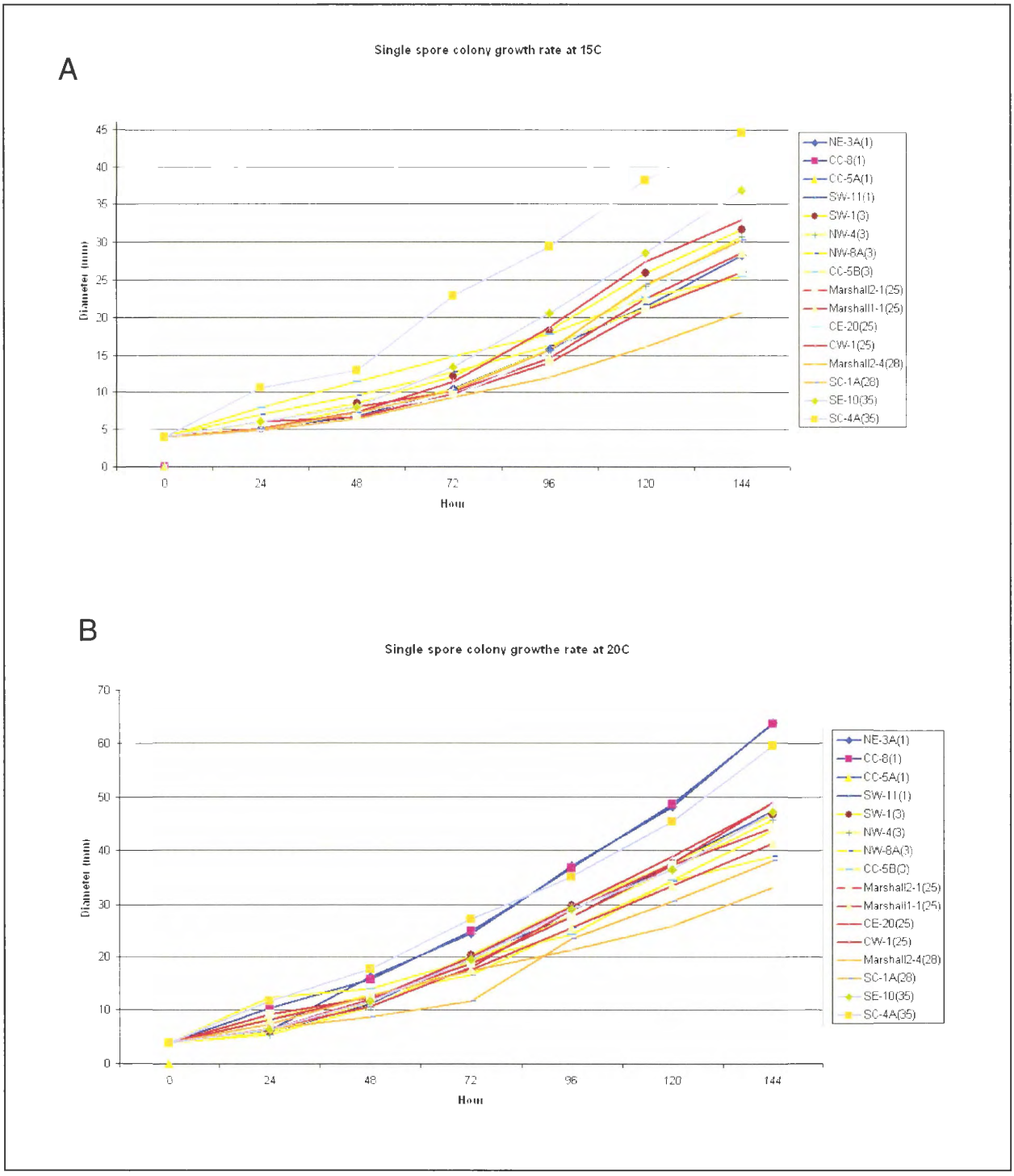

Fig. 3.3. Mycelial growth progress of *P. sojae* in V-8 juice medium at 15°C (A) and 20°C (B). Measurement stopped when the first colony reached the edge of the plate.

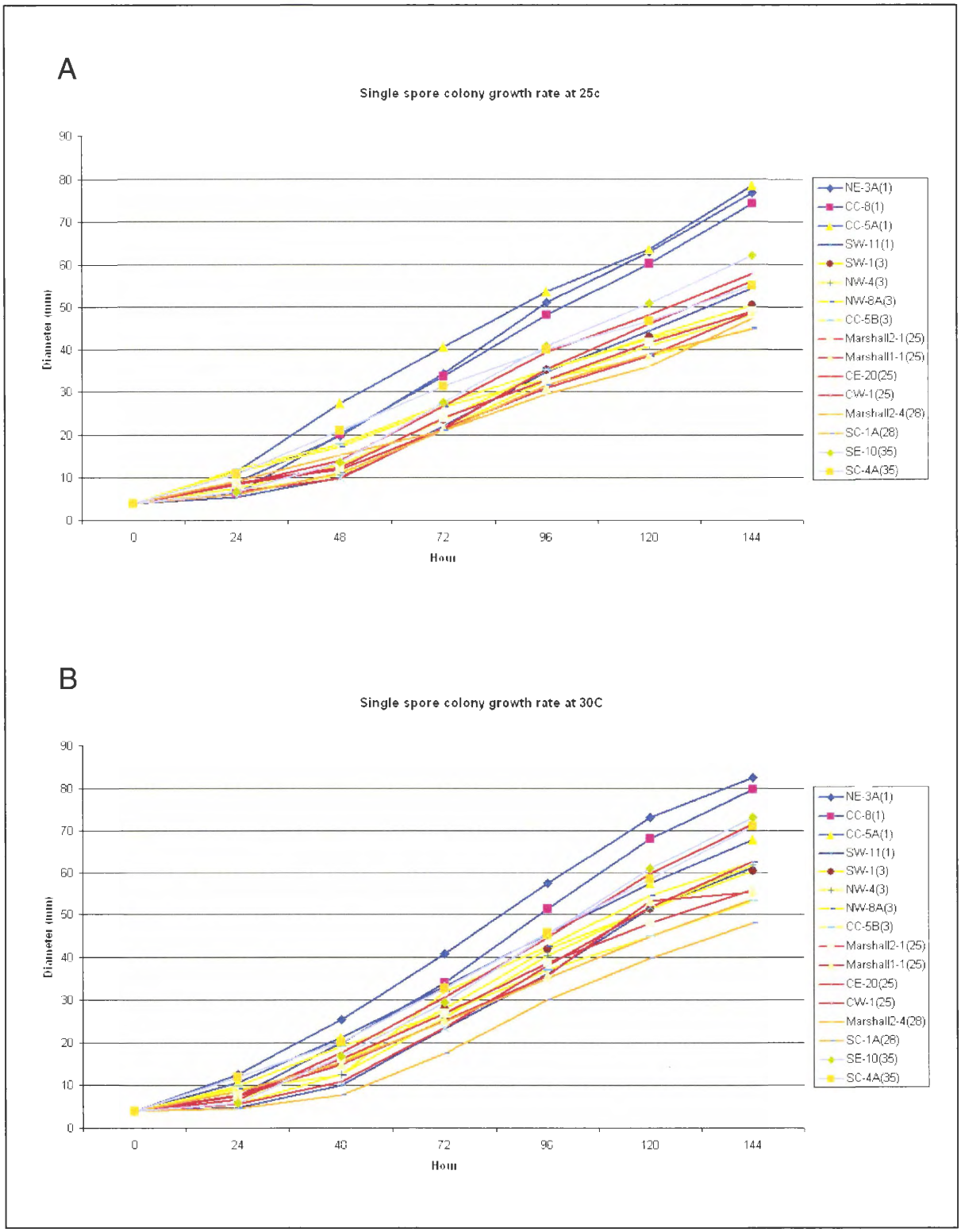

Fig. 3.4. Mycelial growth progress of *P. sojae* in V-8 juice medium at 25°C (A) and 30°C (B). Measurement stopped when the first colony reached the edge of the plate.

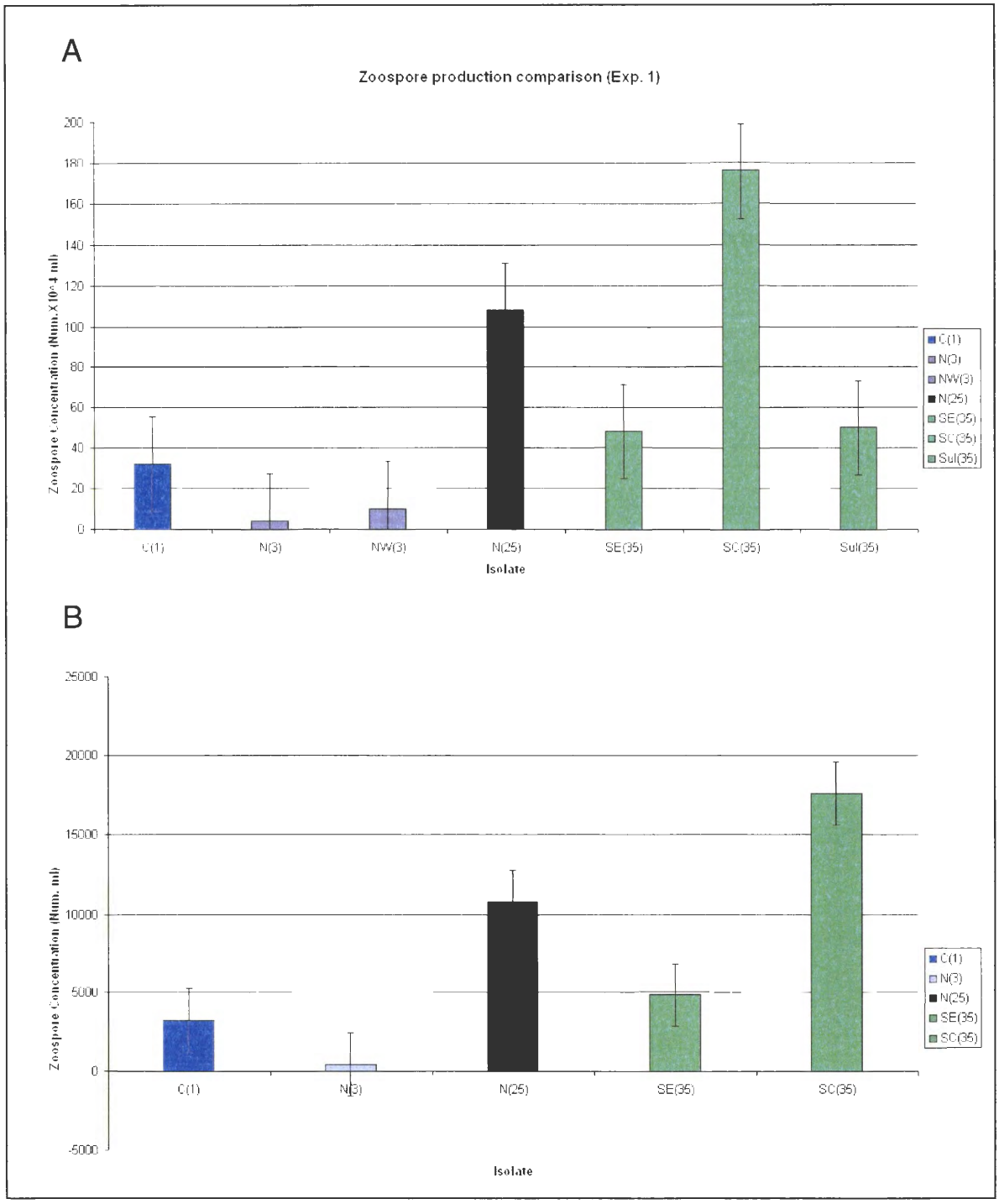

Fig. 3.5. Comparison of zoospore production of isolates from different races of *P. sojae* from preliminary trials. Zoospore production ability was measured by determining the number of zoospore produced by equal amount of mycelia in 1 ml of water and was repeated six times (A) and three times (B).

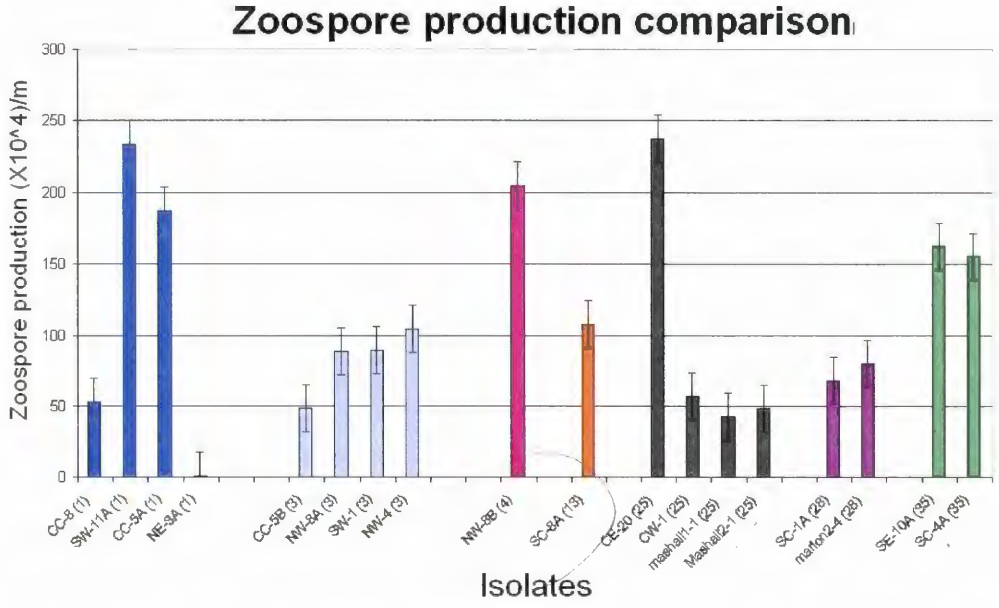

Fig. 3.6. Comparison of zoospore production of isolates from different races of *P. sojae*. Zoospore production ability was measured by determining the number of zoospore produced by equal amount of mycelia in 1 ml of water and was repeated three times.

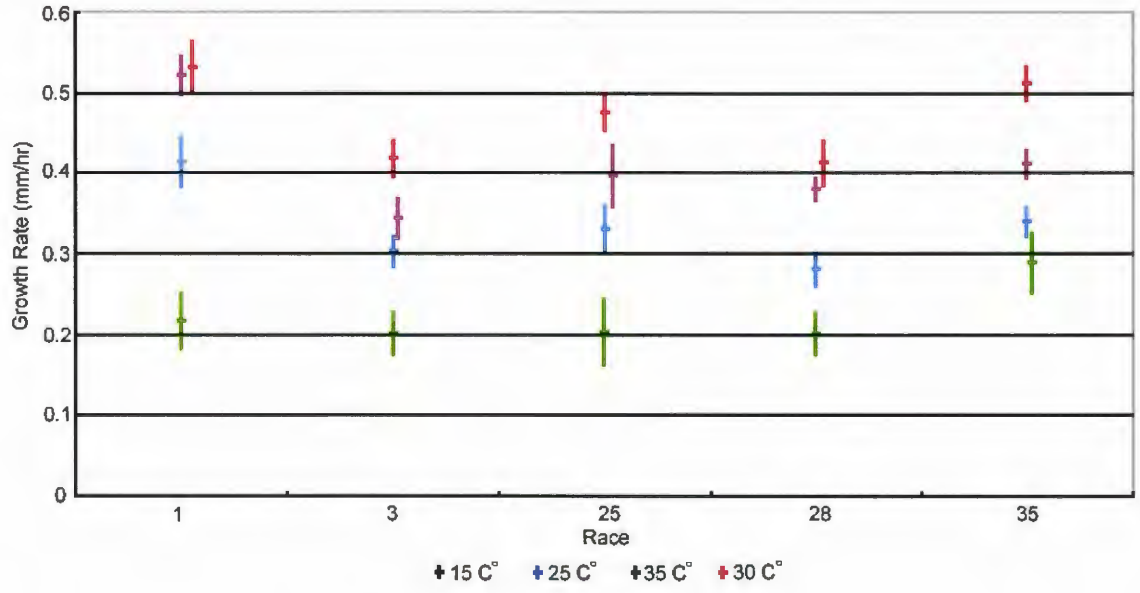

Fig. 3.7. Comparison of the growth rate intervals (with 95% confidence) among *P. sojae* races at 15, 20, 25, and 30°C.

Table 3.1. Comparison of growth rates of different *P. sojae* races under room temperature

Race	Growth rate intervals (95% confidence) (mm/h)	Growth rate intervals (95% confidence) (mm/h)	Difference
1	0.466873	0.584747	A
25	0.339369	0.470511	A B
3	0.084793	0.385627	B
28	0	0.390703	B
35	0	0.47105	B

Growth rates of each race were slopes of colony diameter-time (X-Y) regression lines. The intervals were calculated using standard error with 95% confidence.

Table 3.2. Camparison results of *t* test (LSD) test for latent period of five *P. sojae* races

Race	Mean of latent period (h)	Difference with 95% confidence
3	18.479	A
1	14.740	B
28	14.313	B
35	11.333	C
25	11.302	C

Means with the same letter are not significantly different.

Table 3.3. Results of *t* test (LSD) comparison of the infection incidence of five *P. sojae* races 72 h after inoculation

Race	Mean of infection incidence of different race of *P. sojae* (%)	Difference with 95% confidence
25	73.43	A
1	64.69	A
35	64.38	A B
3	55.94	B
28	51.88	B

Means with the same letter are not significantly different.

Table 3.4. Comparison of *t* test (LSD) test results of HRI (reciprocal of pathogen virulence index [RPVI]) of five races of *P. sojae*

Race	Mean of RPVI	Difference with 95% confidence
25	6.14	A
1	5.43	A B
35	4.45	A B
3	3.44	B
28	3.16	B

Means with the same letter are not significantly different.

Summary

This thesis consists of two parts. In part 1, surveys were conducted to determine the current population of the *P. sojae* race in Iowa soybean fields and the results were compared with those made by Yang *et al.* (1996) 10 yr ago. In part 2, fitness components of selected races of *P. sojae*, including mycelial vegetative growth on media, zoospore production ability, and infection aggressiveness, were evaluated.

In our surveys, 15 isolates from soybean plant samples and 34 isolates from soil samples were recovered in this survey. Among isolates from plant samples, three new races that were not found in Iowa soybean fields during the last survey were identified: race 20, 28, and 35, with percentages of isolation of 13.3, 20, and 6.7, respectively. Eight isolates were found with seven virulence formula that had not been published before (*Rps* 1c; *Rps* 1b, 1d, 3a, 6; *Rps* 1a, 1b, 1c, 1d, 1k, 3a, 7; *Rps* 1a, 1c, 1d; *Rps* 1a, 1b, 1c, 1d, 1k, 6, 7; *Rps* 1a, 1b, 1c, 1k; and *Rps* 1a, 1b, 1d, 1k, 6, 7). These isolates could be new races that have not been reported in the United States. Of plant-derived isolates, 73.3% were virulent to *Rps* 1k in 2001, significantly higher ($P < 0.05$) than the isolation frequency of 4.6% during the survey conducted in 1991, and 1% in 1994. Races that were prevalent during the last survey, such as races 3 and 4, were no longer detected in this study, and they were replaced by races 25 and 28. Among isolates from soil samples, two new isolates were found that had not been reported before this survey, races 28 and 35, with percentages of 2.9 and 11.8, respectively. Races 1 and 3 were the dominant races found from soil samples, with the same detection percentage of 17.6. Isolates that could defeat the *Rps 1k* gene were in 35.3% of the total soil sample isolates, significantly higher ($P < 0.05$) than what was detected during the previous survey (5% in 1992–1993, 2.5% in 1994). Races that are virulent to *Rps* 1k increased their proportion in the *P. sojae* population and replaced the dominant races isolated from plants, whereas the races that were not virulent to *Rps* 1k showed a decreasing trend, but they were still detectable on plants and in soil.

In fitness study, 16 isolates from five selected races that experienced population shifts based on the results from part 1 were tested in this study. Significant differences were found in the fitness components of races 1, 3, 25, 28, and 35. Race 1 had the fastest mycelial growth at 20, 25, and 30°C, and the highest zoospore production ability next to race 35 at room temperature. Its medium length of latent period averaged 14.74 h, and the 64% infection incidence determined that the virulence index value (VIV) was approximately 5.43,

resulting in medium virulence among the five races. Race 25 had a medium vegetative growth rate in all four temperature treatments, medium zoospore production ability, but also the shortest latent period, the highest infection incidence (73%), and the highest VIV (6.14). Race 35 had the highest rate of mycelial growth at 15°C, second fastest to race 1 at the other temperatures. Its overall aggressiveness was weaker than that of race 25 but stronger than that of the other three races. The VIV of race 35 was ~4.4, not significantly different from that of race 1. Race 3 had the weakest fitness characters at all conditions. It was the slowest growing race on the media and produced the least amount of zoospores at room temperature. It had the longest latent period, ~7 h longer than that of race 35, the lowest infection incidence (54%), and the lowest VIV (3.44). Race 28 did not show a significant difference from race 3, but it was slightly weaker than race 3 under all conditions. Generally, all races had strong reproductive ability, and races 25 had the highest infection aggressiveness followed by race 35. Race 1 was in the middle, and races 3 and 28 were at the lowest level. Latent period was the one of the factors that affected the final VIV the most. Races that had shorter latent period had higher VIV as well.

Fitness parameters together with selection pressure resulting from farming could explain the population shift in *P. sojae* observed between this survey and the previous survey. More information is needed for breeders on population monitoring and fitness components of other races of *P. sojae* so that more effective resistant cultivars can be bred to minimize the yield loss caused by *P. sojae*.

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